CHARACTERIZATION OF MCH-I RESTRICTED IMMUNOGENIC PEPTIDES BY IMMUNOLOGICAL AND *IN SILICO* METHODS

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Abstract

The human immune system can provide a powerful tool in developing therapies against various cancers. Even though the idea of an immune system actively searching for and disposing of potential mutated tumor cells is over a century old, only recent developments in various fields such as mass spectrometry, immuno-checkpoint blockade strategies and *in silico* modelling have enabled the realization of the full potential of recruiting immune system to fight cancer and the possibilities of personalized therapies. These therapeutic methods, including but not limited to oncolytic virus therapies, T-cell therapies and cancer vaccines, are based on the body's ability to recognize mutated antigen peptides presented on the cell surface by MCH-receptors (also known as HLA-receptors in humans) and the disposal of the malignant cells by cytotoxic T-cells. Thus, the capability to map the individual HLA-presented peptidome and differentiate the immunogenic peptides is a foundation for this plethora of therapies and is in focus of ongoing research.

This master thesis is a part of a project aiming to set up immunoaffinity-purification/MS based method in order to analyse the ligandome and determine T-cell recognized cancer associated antigens from tumor cells.

Objectives of the work:
1. Characterizing tumor cell lines.
2. Immunological assay set up.
3. Collecting cell culture material for the ligandome affinity purification.
4. *In silico* prediction if the immunogenicity of selected peptides and assessing their source proteins.

Methods used:
1. Cell culture.
2. FACS-analysis.
3. MTS-viability assay.
4. Immunological assays (ELISA, ELISPOT).
5. Immunological bioinformatics analysis tools (IEDB) and database search (UniPROT).

Results:
1. Flow cytometric analysis provided essential information of the cell line HLA-1 expression. Additional information of PD-L1 expression can be used to evaluate cell line's immune-evasion abilities. Preliminary MTS assay is used to determine linear range and optimal time frame for the PBMC/cancer cell co-culture killing assay.
2. Interferon γ cytokine secretion was determined by ELISPOT to assess PBMC response against known antigens in a preliminary experiment to approximate usable range for the following antigen specific PBMC assays. ELISA is used to confirm the presence of HLA-I receptors in the ligandome affinity purification eluates and to estimate the efficacy of purification.
3. Feasibility of in silico methods in the prediction of immunogenic peptides was explored.

The experiments provided information that can be applied to the further development of the immune ligandome discovery project. In silico methods were successfully used to characterize previously identified HLA-restricted peptides and one previously identified immunogenic T-cell epitope. Even if the data acquired *in silico* can be considered only nominally verified at this stage, the results are encouraging.

Keywords

Immunotherapy, cancer, tumor antigen, immunopeptidome, peptidomics, antigen discovery

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Tiivistelmä

Ihmisen immuunijärjestelmä on osoittautunut merkittäväksi tekijäksi kehitettäessä uusia hoitomuotoja erilaisten syöpäsairauksien hoidossa. Vaikka ajatus immuunijärjestelmän aktiivisesta toiminnasta mutatoituneiden syöpäsolujen tunnistamisessa ja hävittämisessä onkin jo yli vuosisadan ikäinen, ovat vasta viimeaikaiset edistysaskeleet useilla aloilla, kuten massaspektrometriassa, T-soluvälitteistä immuunivastetta voimistavissa hoidoissa sekä *in silico* -mallinnusmenetelmissä, mahdollistaneet immuunijärjestelmän kokonaisvaltaisen hyödyntämisen potentiaalin kartoittamisen syöpähoidoissa sekä yksilöllisten hoitomuotojen kehityksessä. Näihin immuterapiamuotoihin kuuluvat muun muassa onkolyyttiset virusterapiat, T-soluvälitteiset hoidot sekä syöpärokotteet. Näiden immunoterapiamuotojen vaikutusmekanismi perustuu elimistön kykyyn tunnistaa mutatoituneiden syöpäsolujen pinnalla esiintyviin MHC-reseptoreihin (tunnetaan ihmisissä nimellä HLA-reseptori) sitoutuneita immuunivasteen herättäviä peptidiantigeenejä, sekä ko. solujen hävittämiseen sytotoksisten T-solujen toimesta. Tästä syystä menetelmät jotka mahdollistavat yksilöllisen HLA-peptidomin tutkimisen sekä immunogeenisten peptidien tunnistamisen tarjoavat perustan immunoterapioiden kirjolle ja ovat jatkuvan tutkimuksen polttopisteessä.

Tämä Pro gradu -tutkielma on osa projektia, jonka tavoitteena on kehittää immunoaffiniteettipuhdistukseen ja nestekromatografia/massaspektrometriaan perustuva menetelmä MHC-ligandien analysoimiseksi ja T-soluvasteen herättävien syöpäantigeenien määrittämiseksi syöpäsoluista.

Työn tavoitteet olivat:
1. Syopäsolulinjojen karakterisointi
2. Immunologisten kokeiden testaaminen
3. Solumateriaalin kasvatus immunoaffiniteettipuhdistusta varten
4. Valikoitujen peptidiligandien immunogeenisyyden arviointi *in silico* -menetelmillä

Käytetyt menetelmät:
1. Solulinjojen kasvatus
2. FACS-analyysi
3. MTS-viebiliteettikokeet
4. Immunologiset kokeet (ELISA, ELISPOT)
5. Immunologisten bioinformatiikka-analyysityökalujen käyttö (IEDB) sekä tietokantahakujen tekeminen (UniPROT)

Tulokset:
1. Virtaussytometria-analyysi (FACS) tarjosi olennaista tietoa tutkittujen solulinjojen HLA-I -ekspressiosta. Ohessa kerätty tieto solujen PD-1 -ligandiekspressiosta voidaan hyödyntää arvioidessa syöpäsolujen kykyä suojautua immuunijärjestelmää vastaan. Alustavia MTS-kokeita käytettiin arvioimaan soveltuvat olosuhteet ja tulosten lineaarisuus jatkotutkimuksia varten.
2. T-solujen interferoni-γ -eritystä tutkittiin ELISPOT-menetelmällä, käyttäen tunnettuja antigeenejä ja koetta käytettiin jatkotutkimusten kehittämiseen. ELISA-menetelmällä varmistettiin HLA-reseptorien läsnäolo immunoaffiniteettipuhdistukseen käytetyistä näytteistä, sekä arvioitiin puhdistusmenetelmän toimivuutta.
3. Tutkittiin *in silico* -menetelmien käyttökelpoisuutta immunogeenisten peptidien tunnistamisessa.

Kokeilla saatiin kerättyä tietoa, jota pystyttiin hyödyntämään projektin ja menetelmien jatkokehityksessä. *In silico* -menetelmillä määritettiin syöpäsoluista joukko entuudestaan tunnettuja MHC-reseptoriin sitoutuvia peptidejä, sekä 1 entuudestaan tunnettu T-soluvasteen herättävä peptidi. Vaikka määritetyt peptidit olivatkin vain nimellisesti validoituja, tulokset rohkaisevat kehittämään menetelmää eteenpäin.

Avainsanat

Immunotherapy, cancer, tumor antigen, immunopeptidome, peptidomics, antigen discovery

Säilytyspaikka

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Muita tietoja

Tutkielman ohjaajat: Karita Peltonen, FT ja Vincenzo Cerullo, FT

Kuvitus Literature Review -osiossa: Lotta Multimäki

CONTENTS

1 INTRODUCTION

"Cancer" is an umbrella term referring to several hundred different types of disease (Hassanpour and Dehghani 2017). It is one of the leading causes of mortality worldwide, but it can be difficult to diagnose and treat efficiently due to the ununiform nature of the disease on a tissue level. Cancer is caused by genetic mutations that result in disturbances in cell function and cell cycle, thus leading to abnormal proliferation. In addition to continuous proliferation, cancer cells are able to avoid apoptosis (NCI a. 2020), or programmed cell death, which is the normal termination point for unneeded or damaged cells. Cancerous cells may grow to form masses of tissue called tumors, either solid or liquid, that are considered benign or malignant. Benign tumors do not invade other tissues, but malignant cancer cells have the ability to invade neighboring tissues or even migrate to proximal tissues through blood circulation or lymph system, forming new tumors. There are several types of cancer treatments, (e.g., surgery, radiation therapy, chemotherapy, immunotherapy) that can be used in combination or as a monotherapy. This work is focused on immunotherapy, a type of therapy that helps immune system to fight cancer.

Cancer causing mutations typically occur in three main types of genes; *proto-oncogenes*, *tumor suppressor genes* and *DNA repair genes* (NCI a. 2020). Proto-oncogenes are responsible for cell growth and division but can become cancer causing genes (oncogenes) when genetic mutations occur. Genetic dysfunction in tumor suppressor genes can trigger uncontrolled cell division. DNA repair genes translate proteins and enzymes involved in locating and fixing damaged DNA (Hassanpour and Dehghani 2017), and mutations in these genes can lead to additional mutations in other genes, disposing individuals to cancer.

Acquired mutations can provide cancer cell populations significant evolutionary advance through diversity and selection, but these mutations also make them susceptible to the body's immune system (Chen and Mellman 2017). Increased mutational burden is associated with efficient recognition as non-self-tissue by immune system and can be used to predict response

to immunotherapy (Samstein et al 2019). The potential of mobilizing immune system for cancer therapy has been recognized over hundred years ago (Coley 1910). However, since cancer cells can develop immune-evasive strategies to escape detection (Chen and Mellman 2017) and because the dynamic interaction between cancer and immune system apparently favors immunosuppressive mechanisms that allow mutated cells to avoid eradication (Palucka and Coussens 2016), more consistent methods, such as radio- and chemotherapy, have dominated cancer therapies (Sharma et al 2017). Despite this lack of early enthusiasm on immunity and cancer, further research pioneered by e.g. Erlich, Burnet and Thomas in the 1950's helped to envision the concept of cancer immunosurveillance and eventually led to realization of the multitude of interactions between tumor cells and immune system. Since then several immune system activation strategies and clinical applications have been developed, such as plethora of cancer vaccines, adoptive cell therapies and immune checkpoint inhibitors.

T-cells are a part of adaptive immune system that play a major part in cancer development (Palucka and Coussens 2016). They can eradicate cancerous cells at the stage of early neoplasia and also fight developed late stage tumors when stimulated by immunotherapy, but they require a unique antigen target to initiate cancer cell recognition and elimination. These targets, initially presented on the cell surface in peptide/MHC-receptor complexes and identified and delivered to T-cells by antigen-presenting cells such as dendritic cells, originate from mutated, viral or overexpressed proteins within the cell and can be classified as tumor associated antigens (TAAs) or tumor specific antigens (TSAs) (Rammensee and Singh-Jasuja 2013). TAAs are self-derived antigens that are typically overexpressed in cancer cell or absent in healthy tissue (e.g. cancer testis antigens). TSAs (also known as neoantigens) are derived for instance from non-synonymous point mutations or viral proteins and ought not to be analogous to other peptides expressed in autologous tissue. TAAs and TSAs presented on tumor cell compose its immunopeptidome, which can be mapped and utilized in immunotherapies in order to achieve greater efficacy and specificity for cancer treatments.

Even if tumor can be assumed to originate from a single founder population, the genetic instability combined with immune system engendered selection pressure results in a set of cancer associated mutations unique to each individual tumor. Accordingly, it is essential to be able to identify these mutations with high precision to achieve individualized and effective tumor antigen selective treatment methods. This master thesis is part of a project aiming to set up protocol to analyze the immunopeptidome and determine T-cell recognized cancer antigens of tumor cells.

Part I. LITERATURE REVIEW

2 Cancer and Immunity

Cancer formation is initiated when healthy tissue cells acquire several features, genetic alterations, that enable them to replicate limitlessly while resisting cell death, edit their imminent microenvironment and invade other tissues (Hanahan and Weinber 2011). Protein expression of these alterations can be presented on the cell surface in form of peptide antigens restricted by class I major histocompatibility complex (MHC-I) receptor, making the cancer visible to the immune system and exposing them to elimination by antigen-specific cytotoxic T lymphocytes (CTL, CD8+ T-cell) (Palucka and Coussens 2016). Cancer tumor arises when cancer cells develop means to evade the immune response. Immunotherapies attempt to re-establish immune systems capability to recognize and effectively dispose of the malignant cells.

For effective T-cell mediated immune response the tumor antigens must be recognized and presented to the naïve T-cells that become activated, locate their tumor tissue and kill the target cells, thus causing the release of additional tumor antigens along with other immune response amplifying signals. This cancer-immunity cycle (Figure 1) is initiated when professional antigen presenting cells, typically dendritic cells residing in the lymph nodes or within tumor tissue, capture the tumor antigen and process it for cross-presentation via MHC-I and MHC-II receptors to CD8+ CTLs or CD4+ helper T-cells, respectively (Shen and Rock 2006). CD8+ cells are responsible to the killing of tumor cells but CD4+ cells help in CD8+ activation and maintenance of enduring response. Primed and active antigen specific CTLs traffic to tumor site and use their T-cell receptors (TCR) to recognize and bind to target cell presenting the antigen within MHC-I complex, eventually killing the cancer cell. Dying cancer cells release additional tumor antigens that may increase the breadth and width of subsequent immune response cycle. Since the antigen presentation by APCs and tumor recognition by CTLs are conducted in association with numerous co-receptors, immune

checkpoints and other either immune response inhibiting or stimulating factors, each of these steps can become cycle limiting.

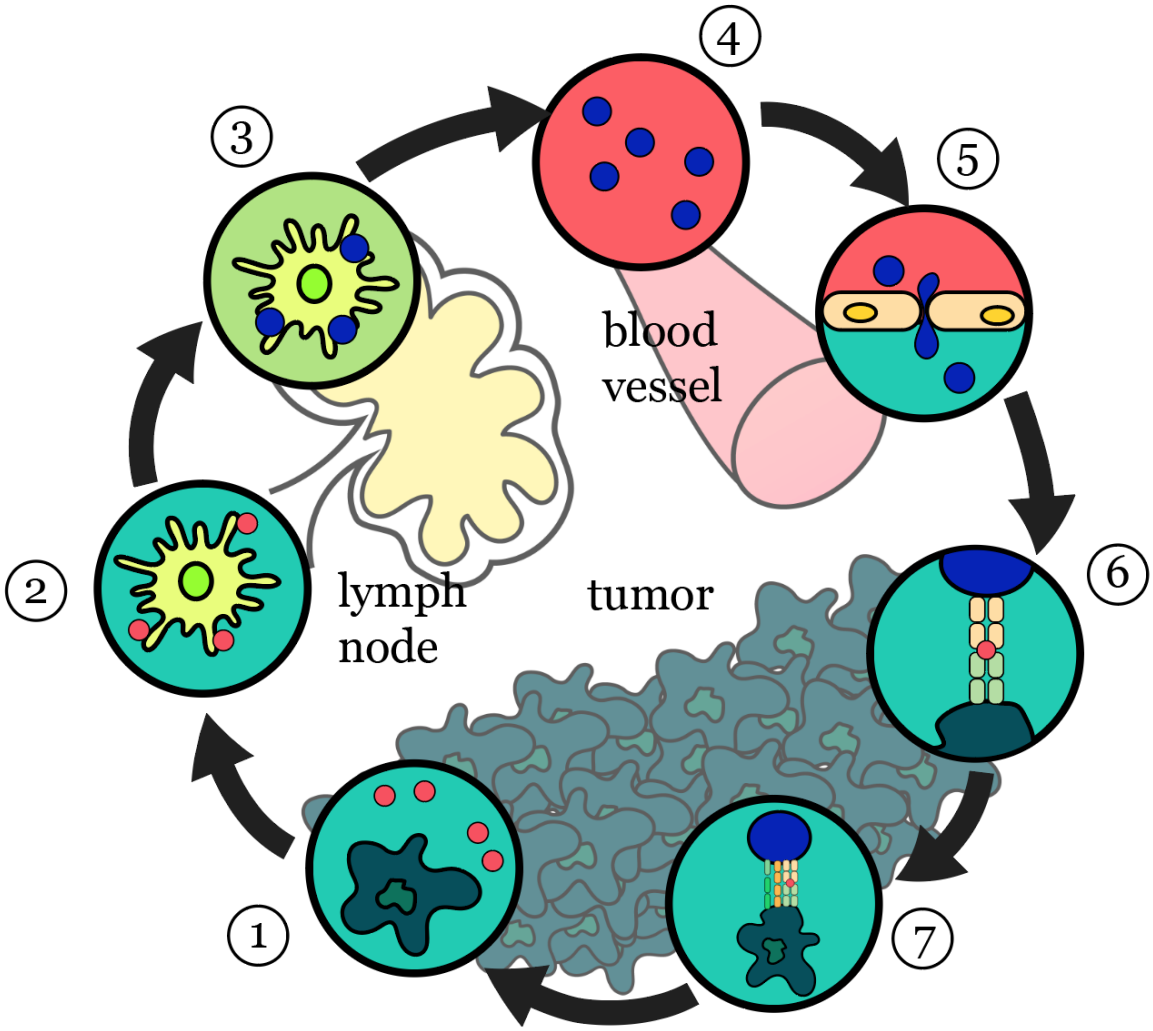

Figure 1: Cancer-immunity cycle. 1. Tumor antigens (red) are released from a dying cell, 2. APC captures antigens and presents them to T-cells (blue) on MHC-I or -II receptors. 3. T-cell priming and activation against specific antigen. 4. Activated T-cells traffic to tumor site. 5. T-cells infiltrating tumor tissue. 6. Recognition of antigen presented on target cell surface. 7. Killing of target cell, that leads to additional release of tumor antigens and completes the cycle.

Melanoma-associated antigen 1 (MAGE-1) was first characterized by van der Bruggen et al. (van der Bruggen et al 1991) in 1991. Since then the number of recognized cancer antigens has increased rapidly, and e.g. Cancer Antigenic Peptide Database

(https://caped.icp.ucl.ac.be/) describes over 450 tumor antigen peptides (Cancerresearch.org 2020). Immunogenic tumor antigen peptides are classified as Tumor-associated Antigens (TAAs) and Tumor-Specific Antigens (TSAs, also referred to as Neoantigens) based on their expression patterns. TAAs are of inherit origin but their expression in cancer tissue differentiates from healthy tissue expression, e.g. cancer testis antigens and overexpressed antigens. TSAs, on the other hand are not expressed in a healthy tissue but are unique sequences and of foreign nature to the body (Murphy 2012).

Even if body has an effective immune system able to recognize tumor antigens and initiate tumor cell death, there are also several mechanisms that can lead to immune-evasion and tumor escape from under the immune surveillance (Palucka and Coussens 2016). Cytotoxic T-cells can be affected by tumor cell's intrinsic immune checkpoint regulators (CTLA-4, PD1) and immunoregulatory cells, such as regulatory T- and B-cells and myeloid cells. Tumor tissue can create microenvironment that can enhance pro-tumor inflammation, attenuate DC cross-presentation of tumor antigens and deplete tumor cell antigen presentation (e.g. MHC downregulation).

When functioning normally the immune cells are selectively recruited into neoplastic tissue and dispose of the damaged cells, but when this fails the preserved neoplastic cells can effectively shape their environment to advance tumor progression through angiogenesis, matrix/tissue remodeling and by recruiting immunosuppressive cells (Hanahan and Coussens 2012). Inflammation seems to have a major role in both tumor development and suppression; chronic inflammation is associated with TH2-type immune response and promotes cancer formation mechanisms, while acute inflammation is linked with TH1-type response and tumor cell elimination. TH2-type cytokine expression profile leads to regulatory T, B-. and myeloid cell activation and increased synthesis of angiogenic, growth and survival factors, all of which are tumorigenic by their nature (Balkwill et al 2005). Macrophage and monocyte activation increase expression of molecules that suppress T-cell proliferation (e.g. arginase 1) and inhibit DC activation and cross-presentation (IL-10) (Ruffell et al 2014). Thus, TH2-type tumor microenvironment is simultaneously immunosuppressive and tumor promoting.

2.1 Major Histocompatibility Complex

Major histocompatibility complexes (MHCs) are receptor proteins that present antigens to the immune system (Murphy 2012). MHC class I receptors bind endogenous antigens and are expressed in almost all types of cells, while MHC class II receptors specialize in binding peptides of exogenous origin and are present only in professional APCs. Third class, MHC III, is not associated with an antigen presenting function. MHC-I molecules (depicted in figure 2.) consist of constant β_2-microglobulin subunit associated with polymorphic α heavy chain that contains the antigen binding groove displayed on cell surfaces (Gires and Seliger 2009). Heavy chains exhibit intense variation with hundreds of known alleles, with each individual able to express up to six different alleles. MHC-I ligand antigens presented by APCs are peptide chains typically 9-10 amino acids long, cleaved and processed from captured immunogenic materials, e.g. virus particles or tumor cell residue. Peptides are bound to pMCH-complex at their amino- and carboxy-terminals, with other residue in the peptide serving as an additional anchor, i.e. anchor residue. The identity and position of anchor residues vary depending on the binding MHC-I allele and changing the anchor affects the MHC receptor binding ability, but also the surrounding peptides can function as secondary anchors and either enhance or limit the pMHC-binding.

MHC molecules are unstable when not binding an antigen but are stabilized when peptide restriction occurs (Murphy 2012). This enables the transportation of pMHC-complex from the endoplasmic reticulum (where peptide processing and binding takes place) to the cell surface, and also allows intact pMHC-complex purification from the cells. MHC class I receptors are stabilized when restricting peptides typically 8 to 10 amino acids long by their amino and carboxy ends. Longer peptides can be bound to MHC I by the carboxy terminus, but these are cleaved enzymatically when the pMHC processing takes place in the endoplasmic reticulum, and resulting peptides presented for T-cell recognition by the MHC class I are typically 9mers in length.

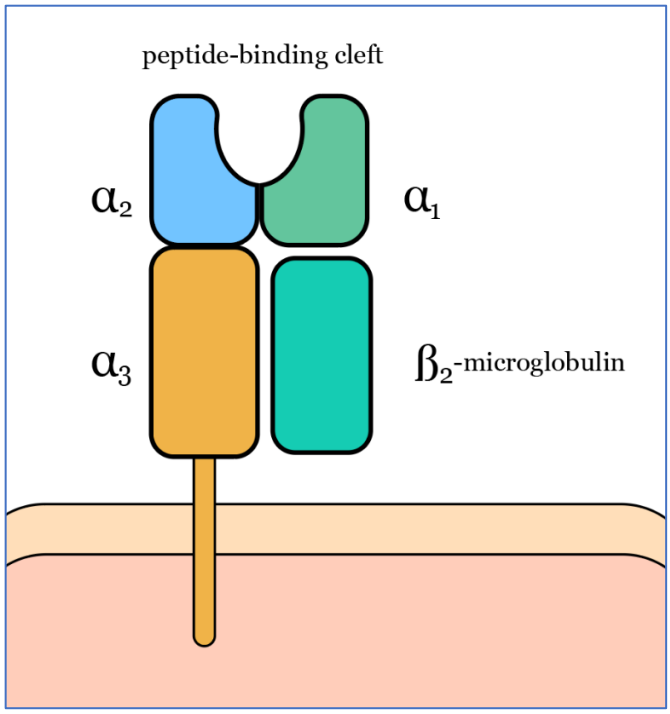

Figure 2: Schematic presentation of the MHC-I heterodimer molecule, consisting of three-domain-α-chain non-covalently bound to β_2-microglobulin. Peptide binding groove is created in the folding of α_1 and α_2 domains.

MHC-II receptor expression is limited to professional APCs (Gires and Seliger 2009). They restrict peptide ligands of 12 to 26 amino acids in length and present them to CD4+ helper T-cells. In general, MHC-I receptors present endogenous peptides and MHC-II are responsible for presentation of peptides of exogenous origin, but both endogenous and exogenous antigens can be presented by both MHC classes through alternative pathways.

MHC class I and class II not only differ by their expression and length, but also how and what type of T-cells they activate (Murphy 2012). Upon antigen presentation and T-cell priming the naïve T-cells bind to peptide-MCH complex by their T-cell receptor (TCR) assisted by co-receptor CD4 or CD8 for helper T-cells and CTLs, respectively (figure 3).

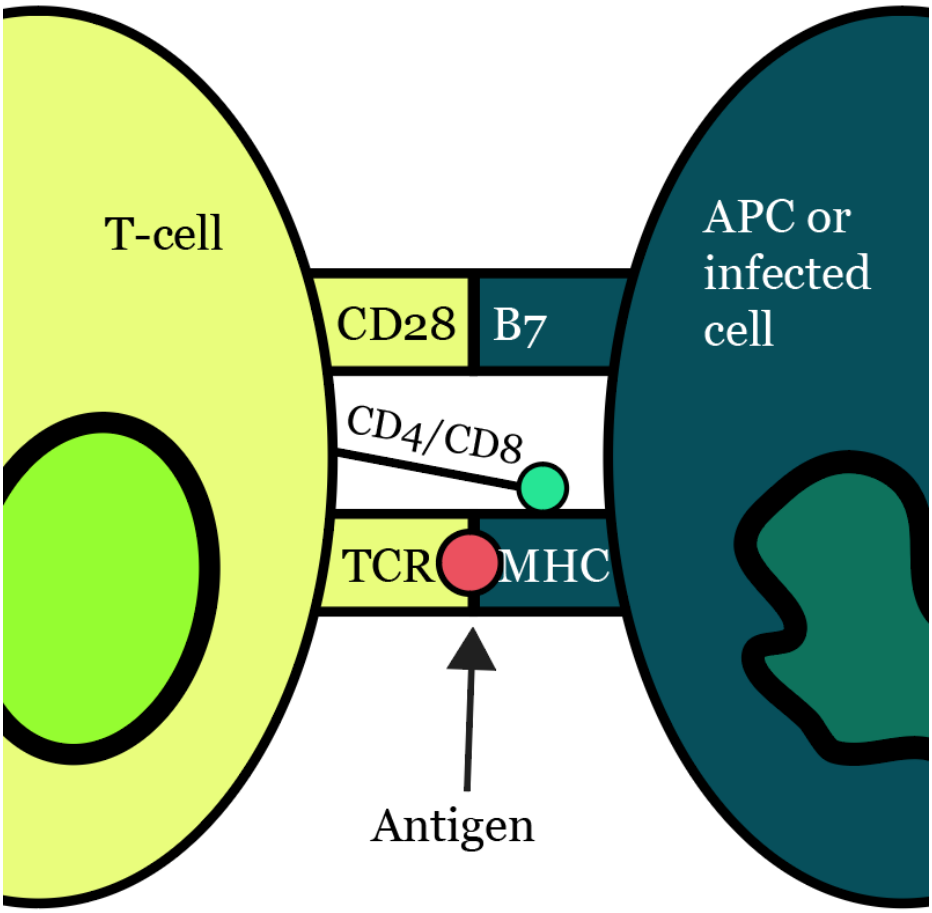

Figure 3: pMHC-I interacting with T-cell receptor and CD4/CD8 co-receptor (for helper T-cells and cytotoxic T-cells, resp.), with additional stimulation provided by T-cell surface receptor CD28 binding to APC specific B7-ligand (Smith-Garvin et al 2009). Several other receptors and signaling are involved in T-cell activation, but none provide as strong stimuli as CD28, which also prevents unintentional activation by non-professional APCs (Murphy 2012)

2.2 Cancer immunotherapies

A wide assortment of therapies of different types are used as cancer treatments (NCI a 2020). The selected therapy depends on the type of the cancer and the stage of the cancer development, and they can be used as a monotherapy or combination therapy. Variety of

treatments ranges from established types that have been in use for a long time, such as surgery, chemotherapy, and radiation therapy, to more recent approaches in cancer treatment such as immunotherapies. Immunotherapy is a type of biological therapy, a type of treatment that uses substances made from living organisms to treat cancer (NIH 2019). There are several types of immunotherapies, and here are presented some of them, based on either their utilization of MCH-antigen presentation or their overall significance in cancer treatment, e.g. immune checkpoint inhibitors.

2.2.1 Cancer vaccines

Certain viruses, such as Human Papilloma Virus (HPV), predispose tissues to tumor formation (Villa et al 2007). Vaccinating against these oncogenic viruses is a form of prophylactic cancer vaccination, since suppression of the viral infection also indirectly prevents associated cancers. Prophylactic cancer vaccines have shown efficacy against various tumors in animal models (Finn and Forni 2002) and in clinical practice as anticancer immune therapy in humans (Villa et al 2007).

The other type of cancer vaccinations are therapeutic cancer vaccines, aimed to induce immune response against tumors already present in body. These include antigen-specific-, dendritic cell- and whole cell vaccines enhanced with cytokine-based adjuvants. Antigen-specific vaccines utilize antigenic proteins or peptides to generate cell mediated immune response, target antigens being i.e. cancer testes antigens, such as MAGE-A3 (Atanackovic et al 2008) and NY-ESO-1 (Valmori et al 2007), that are typically abundant in certain cancer tissues or other proteins overexpressed in tumors, like HER2/neu (Disis et al 2002) in breast cancer subtypes.

In dendritic cell vaccination immature dendritic cells are harvested from the patient, simulated with antigen peptide or protein and transplanted back into the patient where they

induce specific immune response by presenting target antigen to T-cells (Schuler et al 2003). Harvested dendritic cells are activated by co-stimulation with inflammatory cytokines or microbe recognition patterns receptor agonists (Napolitani et al 2005), in order to promote antigen presenting abilities, expression of T-cell costimulatory proteins and to increase dendritic cell migration into lymph nodes where antigens are presented to naïve T-cells. The first FDA approved therapeutic cancer vaccine Sipuleucel-T (Cheever and Higano 2011) was designed as a vaccine composed of antigen/co-stimulant loaded matured dendritic cells, even if the final product also contains other types of peripheral blood mononuclear cells (PBMCs).

Cytokines such as IL-2, IL-12, IFN-α and GM-CSF have been studied as adjuvants to increase the efficacy of cancer vaccines (Berinstein 2007). In addition to being used as an adjuvant GM-CSF is also incorporated in autologous tumor cell (ATC) vaccination strategy GVAX (Soiffer et al 1998). In ATC vaccine cancer cells are processed into a vaccine formulation to prevent their proliferation and to provide the immune system with numerous patient specific tumor associated antigen targets. GVAX utilizes genetically modified, GM-CSF secreting autologous tumor cells in order to induce amplified and coordinated B- and T-cell response against wide variety of epitopes simultaneously. GVAX can also be used in combination with checkpoint inhibitors to treat melanoma (Curran et al 2010) and is currently (at autumn 2019) in clinical trials to treat metastatic renal cell carcinoma (Koster et al 2019) and Pancreatic cancer (NIH 2019).

2.2.2 Oncolytic viruses

Oncolytic Virus refers to several types of viruses, in example Vaccinia (Stojdl et al 2003), Poxvirus (Parato et al 2011), Herpes Simplex virus (Martuza et al 1991) and Adenoviruses (Kanerva et al 2013) (amongst several others), that have the ability to selectively infect and cause cell death in cancer cells. Certain cancer types overexpress receptors that mediate virus invasion, for example CD46 surface receptor that inactivates the complement pathway and prevents cell death in cancer cells (Anderson et al 2004) is also the entry receptor for

Edmonston strain measles virus (Dorig et al 1993). Also, Herpes Simplex virus 1 (HSV-1) (Yu et al 2005) and coxsackievirus (Guo et al 2014) use cell receptors upregulated in cancer as a port of entry in various tumors.

Even if oncolytic viruses might be able to infect both normal and cancer cells, cancer cells can be more susceptible for virus infection because of malfunctions in inherent antiviral functions (Hanahan and Weinberg 2011). For example, in case of virus infection healthy cells release interferons which in turn activate Protein Kinase R (PKR) production (Elde et al 2009). PKR in turn is able to detect presence of double stranded RNA and other viral elements and sequentially terminate protein production and initiate cell death. In cancer cells these pathways and viral clearance may be dysfunctional (Clemens 2004). Other possible factor limiting virus infection to cancer cells is the p53 protein pathway, which induces apoptosis and permits virus clearance in healthy cells but is often defective in tumor cells (Takaoka et al 2003).

Besides the oncolytic effect also the tumor vasculature (Breitbach et al 2013) and blood flow (Breitbach et al 2007) can be disrupted, but the primary antitumoral effect is apparently the viruses' ability to stimulate and target immune system against cancer cells (Liu et al 2003), thus functioning as an in situ anti-cancer vaccination. This is based on the tumor antigens released by the dying cells, that are presented to T cells by antigen presenting cells (Moehler et al 2005). Upon cell death in addition to tumor antigens also viral pathogen associated molecular pattern signals (PAMPs), danger associated molecular pattern signals (DAMPs) and cytokines are being released, leading to innate (NK cells) and adaptive immune response (cytotoxic T cells) and tumor rejection also at distant tumor sites (Zamarin et al 2014). Furthermore, oncolytic viruses might be effective in overcoming tumors' immune suppression strategies. IFN and DAMP stimulated NK cells can detect and destroy cells with downregulated MHC-I expression, a common immune evasion mechanism in cancer (Ljunggren and Karre 1985). Oncolytic viruses can also alter the immunosuppressive microenvironment created by tumor (Di Paolo et al 2009; Prestwich et al 2009), enabling recognition and eradication.

Recombinant techniques can be used to improve virus's oncolytic selectivity (You et al 2001), enhance cytokine-based stimuli (Burke et al 2012) and to induce targeted anti-cancer response by tumor antigen expression (Vigil et al 2008). Selectivity is improved e.g. in Ad5/3Δ24 adenovirus that has been modified to bind to integrins overexpressed in ovarian cancer (Liapis et al 1997), adenovirus ONYX-015 that can only replicate in p53 deficient cells (Heise et al 1997) and in attenuated HSV-1 that can reproduce only in cells with defective PKR pathway (Poppers et al 2000). As in cancer vaccines such as GVAX, oncolytic viruses can be modified to express GM-CSF as an adjuvant to provide additional immune stimulation (Burke et al 2012). FDA approved oncolytic virus therapy Talimogene Laherparepvec (T-VEC) also encodes for GM-CSF (Andtbacka et al 2015). Oncolytic viruses modified to express tumor antigens have been studied to improve selective antitumor immunity (Sorensen et al 2009) but coating oncolytic virus with desired antigen peptide might provide a more flexible platform for personalized cancer therapy (Capasso et al 2016), (Ylösmäki et al 2018).

Oncolytic virotherapy can be adapted to other cancer therapies, such as immune checkpoint inhibition (Zamarin et al 2014; Feola et al 2018) and DNA vaccine (Lopes et al 2019). So far one oncolytic virus therapy, T-VEC, has been approved by FDA in treatment of patients with advanced melanoma (FDA 2015). T-VEC is modified to selectively infect PKR deficient cells, to limit its ability to grow and induce latent infection in neurons, and to express GM-CSF to improve antitumor response (Andtbacka et al, 2015).

2.2.3 Adoptive Cell Therapies

Adoptive Cell Transfer (ACT) therapy refers to a type of immunotherapy where T-cells are administered to a patient (NCI b. 2020). T-cells utilized in ACT are Tumor-Infiltrating Leukocytes (TILs) harvested from the tumor tissue, T-cells with genetically modified T-Cell Receptor (TCR T-cells) or Chimeric Antigen Receptor T-cells (CAR-T cells) modified to recognize tumor-specific antigens on cancer cells. Major divergence between these is that T-

cell receptors restrict MHC bound peptides presented by APCs, whilst CAR-T cells recognize non-pMCH antigens on cancer cell surface (Gross et al 1989).

The efficacy of early attempts to utilize T-cells against cancer was compromised by the technical difficulties in T-cell cultivation *in vitro* (Delorme et al 1694; Fefer et al 1969). Nevertheless, it was later demonstrated that melanoma derived TILs were able to recognize autologous tumors (Muul et al 1987) and mediate cancer regression (Rosenberg et al 1988). In general, administered T-cells were short lived and response duration was limited, but combining TIL therapy with prior chemotherapy resulted in clonal repopulation with the transferred T-cells and durable response (Dudley et al 2002). Lymphodepletion by chemotherapy induces T-cell growth factor IL-15 production, which in turn promotes the expansion of transferred cells without competing endogenous lymphocytes (Dudley et al 2008). So far TIL therapy has shown consistent results on metastatic melanoma only (Humphries et al 2013), possibly due to the immune tolerance associated with autologous TILs pre-exposed to TAA derived pMHCs (Staveley-O'Carroll et al 1998) and problems related to successful harvest of primed TILs from different histologies (Humphries et al 2013). It is also suggested that TIL therapy efficacy is associated with high mutational load of metastatic melanoma (Gubin et al 2014).

In order to achieve a wider spectrum of therapeutic applications, lymphocytes collected from peripheral blood circulation (instead of harvesting primed TILs from tumor sample) can be modified to target specific antigen presenting pMHC, such as HLA-A2 restricted melanoma-melanocyte antigen MART-1 (Morgan et al 2006). This enabled higher recognition rate of cells with lower MART-1 expression by the TCR T-cells, but also resulted in on-target, off-tumor toxicity targeting healthy melanocytes in other organs (Johnson et al 2009), also expressing melanoma melanocyte differentiation proteins MART-1 and gp100 (Kawakami et al 1994a; Kawakami et al 1994b) recognized by TILs, but previously unaffected by TIL therapy.

TCR cells can recognize cells expressing miniscule amounts of pMHCs and can be triggered by only one such target complex (Sykulev et al 1996). Thus, engineered TCR T-cells with

high affinity towards self-derived TAAs create a risk of toxicity on healthy tissues (Holler et al 2003). There is also a risk of unexpected cross-reactivities against similar epitopes of different origin, as displayed by targeting cancer testes antigen MAGE-A3 (Morgan et al 2013) that led to recognizing unrelated epitopes in the CNS and in the cardiac muscle (Linette et al 2013). Nevertheless, cancer testis antigen NY-ESO-1 reactive TCR therapy has so far provided signs of clinical efficacy without off-target/off-tumor toxicity. Considering that apparently target recognition efficacy isn't in direct relation to target affinity to T-cell receptor (Valitutti et al 1995) and due to potential off-tumor reactivity, target antigen selection (e.g. targeting TSAs instead of TAAs) and TCR affinity adjustment must be carefully optimized to avoid potentially fatal adverse effects. TCRs fitted with an apoptosis inducing molecular kill switch can also be used to control the risk of toxicity (Di Stasi et al 2011).

CAR-T cells were initially developed in the late 1980's (Gross et al 1989). They are composed of variable regions of antigen binding extracellular antibody chains, linked to T-cell activating intracellular domain. CAR-T cells recognize their target antigen in an MHC-independent fashion by binding to extracellular surface structures instead of intracellular MCH restricted antigens. First generation CAR-T cells were found lacking in efficacy and longevity *in vivo* (Till et al 2008) but second-generation CARs, incorporating co-stimulatory signaling components (Imai et al 2004), displayed improvements in antitumor efficacy and persistence (Song et al 2011).

CAR-T therapy targets must be carefully selected in order to achieve sufficient efficacy while avoiding off-target adverse effects. Ideal target molecules are expressed in cancer cells in such amounts that allow CAR-T activation, e.g. at least several hundred molecules/cell in case of B-lymphocyte antigen CD20 (Watanabe et al 2015), but aren't present in essential organs. B cell antigen CD19, required for B cell development, is expressed in high levels in several B cell malignancies but is not displayed in other tissue (van Zelm et al 2006). CAR-T therapy achieved first clinical success in treatment of advanced B cell lymphoma (Kochenderfer et al 2010) and has since been applied in treatment of several other B cell

cancers, such as acute lymphoblastic leukemia (Grupp et al 2013) and large B cell lymphoma (Kochenderfer et al 2015) Developing CAR-T therapy applications for cancers outside B cell lineage has proven difficult, because of either fatal on-target/off-tumor toxicity (Morgan et al 2010; Lamers et al 2013; Thistlethwaite et al 2017) or lack of efficacy (Ahmed et al 2015). Even in CD19 CAR-T therapy B cell aplasia causing on-target toxicity and excessive cytokine release are serious potential adverse effects (Lee et al 2014). By targeting extracellular structures CAR-T therapy avoids the risk of tumor escape by MCH downregulation but nevertheless, cancer resistance due to antigen loss is still a possibility (Sotillo et al 2017).

ACT utilizing TCRs has displayed efficacy in certain solid tumors (Robbins et al 2011; Robbins et al 2015) but CAR-T based ACTs in solid tumors have so far been ineffective (Lamers et al 2013) regarding tumor control but pose a risk of off-tumor toxicity. The lack of effect is probably caused by immunosuppressive microenvironment observed in numerous solid tumors (Rabinovich et al 2007), while off-tumor toxicity can be due to large amount of target molecules required for CAR-T activation (as compared with TCR T-cells) that leads to toxicity in healthy tissue expressing same target molecule in commensurate amounts. It might prove beneficial to utilize multiple antigen targeting ACT in tandem with immune checkpoint inhibitor therapy in order to avoid selection-based tumor escape and to de-emphasize tumor immune-evasion strategies.

2.2.4 Immune checkpoint inhibitors

T-cell activity can be downregulated by Immune Checkpoint pathways, such as cytotoxic T lymphocyte–associated protein 4 (CTLA-4) outcompeting costimulatory CD28 signaling required for T-cell activation, and programmed cell death 1 (PD-1) receptor interacting with programmed cell death ligand 1 (PD-L1). Immune checkpoints are a part of the immune system responsible for preventing the immune system from attacking healthy cells and tissue

(NCI c. 2020), but cancer cells can exploit this mechanism to avoid immune response and destruction by T-cells.

CTLA-4 Immune Checkpoint pathway was discovered when it was demonstrated that upon T-Cell Receptor activation the intracellular CTLA-4 protein translocates to the cell surface and inhibits CD28 mediated T-cell proliferation and activation (Chambers et al 2001). It was hypothesized (Leach et al 1996) that blocking CTLA-4 proteins with antibodies would lead to more efficient T-cell activation and establish durable anti-cancer response. First CTLA-4 inhibitors, Ipilimumab and Tremelimumab, could indeed induce a long-term tumor regression, especially in some advanced metastatic melanoma patients lasting over ten years after the therapy (Schadendorf et al 2015). Unfortunately, CTLA-4 blockade therapy could be associated with inflammatory adverse events such as enterocolitis, dermatitis, and thyroidal inflammation (Hodi et al 2003; Ribas et al 2005, Postow et al 2018), but with relatively low antitumoral response rate. In general, CTLA-4 inhibition therapy of tumors with a high mutational burden seems to be associated with greater likelihood of response (Snyder et al 2018).

Programmed Cell Death 1 receptor (PD-1) was initially found to induce cell death in T-cell hybridoma (Ishida et al 1992) but was later recognized as an Immune Checkpoint with cytotoxic T-cell inhibitory effect (Baumeister et al 2016). PD-1 expressed on the surface of CTLs has two ligands, PD-L1 being expressed in various somatic cells following proinflammatory exposure and PD-L2 with an expression profile limited to APCs. The PD-L1 expression in cancer cells can have a major role in inhibiting the T-cell mediated antitumor response, when PD-L1 expression in tumor cells leads to exhaustion of tumor recognizing T-cells (Sen et al 2016). TCR activation in tumor recognizing CTLs induces interferon-g (IFN-g) proliferation, which in turn stimulates PD-L1 expression in cancer cells (Baumeister et al 2016). Thus, the PD-1 pathway inhibition leads to much more defined antitumoral response, since the PD-1/PD-L1 blockade acts on already activated, tumor specific CTLs. This can lead to pronounced therapeutic effect with more limited toxicity than CTLA-4 inhibition therapy (Wolchok et al 2017), which was demonstrated in the first clinical

trial with PD-1 inhibiting Nivolumab when six of the 16 patients receiving the treatment experienced tumor response with limited toxic effects (Topalian et al 2012).

Data indicates that patients typically respond to anti-PD-1/PD-L1 treatment because of pre-existing antitumor response by tumor infiltrating CTLs. In such cases when the tumor has adapted by expressing PD-L1s to inhibit CTLs, the PD-1/PD-L1 inhibition treatment re-enables the CTLs to act against tumor cells (Blank et al 2016). Again, the tumor types with high mutational burden are associated with CTL infiltrations and robust antitumoral response, but it seems that there is variability in mutation types' sensitivity to immune checkpoint inhibition therapy; Early mutations in tumor founder cells carried on to the clonal cells are related to better response (McGranahan et al 2016), while belated subclonal mutations are not as sensitive to PD-1/PD-L1 inhibition. Naturally, how the immunogenic peptides resulting from the mutations are presented by the MHC-receptor affects the CTL recognition and antitumor response (McGranahan et al 2017). Thus, there is a possibility that anti-PD-1/PD-L1 therapies might benefit when used in combination with other immunologic therapies that enhance antigen presentation to T-cell mediated immune response (Feola et al 2018).

2.3 Immunotherapy targets

2.3.1 Tumor associated antigens

Tumor-Associated antigens can be subclassified into groups such as cancer-testis antigens, oncofetal antigens, differentiation antigens, differential posttranslational modified antigens or overexpressed self-antigens (Di Marco et al, 2017). TSAs are sometimes also referred as Shared Tumor Antigens, since they are expressed in a large fraction of tumors of a certain cancer type. Even if they are of self-origin and sometimes tolerated by the immune system, many are also immunogenic and T-cell responses against TAAs are often correlated with

patient survival. It is possible that immunogenicity of TAAs is not the limiting step (Vermeij et al, 2011), but tumor immunosuppressive microenvironment might pose a greater issue for successful immunotherapy (Palucka and Coussens, 2016). Some examples of recognized TAAs are described below.

Cancer testis antigens, caused by demethylation occurring in a tumor cells, are normally expressed in MHC-negative testicular germ cells and placental trophoblasts but not in significant number in other healthy tissue (De Smet et al, 1999). They are present in many tumors of different origin and because of this restricted expression pattern might be also classified as Tumor Specific Antigens, but since they have been found to be expressed in e.g. medullar thymic epithelium cells the possibility of self-tolerance cannot be excluded (Kyewski and Klein, 2006). MAGE antigen family (Atanackovic et al 2008) and NY-ESO-1 (Valmori et al 2007) are well known cancer testis antigens investigated in several clinical trials for cancer immunotherapy.

Differentiation antigens are expressed only in specific tissue types (Murphy 2012), e.g. in melanocytes and melanoma cells. Because these TAAs are present in both normal and tumor tissue they are considered low-specificity antigens and both central and peripheral tolerance mechanisms prevent the action of specific T-cells (Kyewski and Klein, 2006). Nevertheless, the self-tolerance for differentiation antigens can be incomplete and some melanoma differentiation antigens can induce specific T-cell response. Well known examples MART-1, gp100, TRP-1 and TRP-2 (Robbins et al, 2002), (Sun et al, 2000) are targeted by helper T-cells and CTLs. CD20 is also a well-known B-cell differentiation antigen that has been studied in B-cell lymphoma therapy dual targeting CD19/CD20 (Schultz and Mackall 2019).

Some antigens are strongly overexpressed in the tumors compared to the normal tissue (Murphy 2012). HER-2/neu is overexpressed in several adenocarcinomas, such as breast cancer (Disis et al 2002), and recognized by CTLs. T-cells specific to overexpressed antigens typically ignore in vitro normal cells that express the antigen in question, but in vivo tolerance mechanisms act against the T-cells anti-tumor activity (Kyewski and Klein, 2006). In case of

hundred-fold overexpression of a given antigen, this self-tolerance can be circumvented (Zinkernagel and Hengartner, 2001).

2.3.2 Tumor specific antigens

Tumor Specific antigens, also known as Neoantigens, are defined by National Cancer Institute as “a new protein that forms on cancer cells when certain mutations occur in tumor DNA”, and they may play an important role in helping the body make an immune response against cancer cells (NCI d. 2020). As the name “tumor-specific” implies, they are unique molecules without autologous expression in heathy tissue (Di Marco et al 2017) and thus in principle provide ideal targets for cancer immunotherapies. The singular character of the TSAs also ensures that T-Cells recognizing TSAs are unaffected by the immune systems tolerance mechanisms (Lennerz et al 2005). Because of the mutation-prone nature of cancer formation there is a high probability that each tumor expresses several tumor-specific antigens.

Unfortunately only a minor amount of cancer specific mutations lead to the formation of MHC-restricted immunogenic peptides (Di Marco et al 2017), and even in melanoma, which has a high mutational burden, the occurrence of T-cell response inducing TSAs can be considered rare. In addition, most of TSAs are patient specific, that complicates the development of “off-the-shelf” treatments and require personalized therapy approach. Also, if the TSAs are derived from passenger mutations (not essential to cancer progression) they might provide a poor target for immunotherapies and lead to immune evasion. Nevertheless, in some tumor types there are common unique antigens, where the expression is attributed to shared biological function essential to tumor formation (Lennerz et al 2005). In some cases, the viral origin of the cancer can lead to shared TSA expression, e.g. B-cell malignancies resulting from Epstein Barr Virus infection (Lee et al 2004).

2.4 Tumor antigen identification

The technical aspects of immunopeptidomics (evaluation of mass spectrometry methods, targeted MS etc.) and the comprehensive account for process of neoantigen discovery are well beyond the scope of this master thesis but basic principles of current neoantigen pipelines are introduced, along with immunopeptidome identification protocol described by MS-based antigen discovery pioneer Hans-Georg Rammensee (Di Marco et al 2017).

2.4.1 Immunopeptidomics

Neoantigens provide a tumor specific target for immunotherapy but do not necessarily evoke immune response even in tumors with high mutational load (Linnemann et al 2015). Also, if the antigen is derived from a passenger mutation that is non-essential for the tumor development immune evasion might occur. Electing tumor associated antigens (e.g. cancer testis antigens) over tumor specific neoantigens may avoid these issues by providing targets that are potentially immunogenic and also possibly commonly shared within tumors of similar cancer type (Di Marco et al 2017). Immunopeptidome analysis can be performed on any tissue with MCH expression but required sample size is MHC expression dependent and ranging from approximately several hundred milligrams to 1 gram (Bassani-Sternberg and Coukos 2016).

For the characterization of immunopeptidome expressed by certain cell line or tissue, the MCH ligands must be isolated from the sample, and the most common methods applied for this purpose are mild acid elution and immunoaffinity purification. The use of mild acid elution is limited to single-cell suspensions (Storkus et al 1993) and MHC-I ligands. Immunoaffinity purification method utilizes MHC-specific antibodies to capture pMHC complexes from cell lysate (Falk et al 1991). IP method is usable for both single cell suspensions and tissue samples, it can be applied to both MCH-I and MCH-II classes and the

results are less prone to contain contaminants compared to mild acid elution method (Fortier et al 2008; de Verteuil et al 2010). Approximately at least 10 000 unique peptide molecules are present in any sufficient tissue sample, healthy or cancerous (Hillen and Stevanovic 2006).

Isolated ligands are thereafter identified by using high performance liquid chromatography and mass spectrometry-based methods. LC-MS/MS was first applied for the identification of viral MHC peptide ligands since 1990 (Rötzschke et al 1990) and for autologous peptides since 1992 (Hunt et al, 1992). Tumor antigens can be recognized by comparing tumor immunopeptidome to a healthy tissue sample (Rammensee et al 2002), already known tumor associated antigens and their corresponding source proteins can be identified by interrogating public protein database such as UniPROT, and ligands' MHC-receptor binding ability can be validated using in vitro binding assays (Gfeller et al 2016). Finally, the antigen immunogenicity is assessed and immune response inducing T-cell epitopes validated by T-cell based assays.

2.4.2 Neoantigen discovery

Current neoantigen discovery pipelines are initiated by locating genomic alterations in cancer cells (Gfeller et al 2016). Whole genome sequencing or whole exome sequencing are used to compare tumor DNA and DNA from healthy tissue to reference human genome to identify non-synonymous mutations (NSM, mutations affecting the protein amino acid composition) occurring in tumor. This is done using variant calling algorithms (Xu 2018) and since different algorithms produce inconsistent results the discovery pipelines use typically several complementary variant callers to avoid false positives. RNA sequencing can complement DNA sequencing to filter out neoantigen candidates without expression (Bassani-Sternberg and Coukos 2016).

Identified mutated peptide sequences are ranked by their MHC-allele binding quality utilizing in silico prediction methods (Bassani-Sternberg and Coukos 2016). These machine

learning algorithms have been trained to evaluate MCH receptors ability to bind specific peptides by large datasets of verified ligands. Binding prediction process delivers a list of hundreds of potential MHC-binding peptides, so because of current limitations in immunogenicity screening methods the number of ligands must be narrowed down. Prediction algorithms present results for strong MHC binders as an "absolute" IC50 value or percentile rank score relative to large set of random peptides (Jurtz et al 2017). Using suitable threshold values such as IC50<500nm or percentile rank score ≤2 provides a feasible amount of ligands for neoantigen screening assays. It is essential to recognize that strong binding capability does not necessarily equate T-cell activation (a phenomenon known as prediction bias).

Alternative pipeline for neoantigen selection is to use MS-based immunopeptidome analysis (see 2.4.1 Immunopeptidomics) of tumor sample and match acquired data to a customized database generated by genomic sequencing (Yadav et al 2014). This can provide a list of MCH-restricted peptide sequences (usually less than 10) for validation and further immunogenicity assay. Advantages of this method is that the list of neoantigen candidates and the number of false positives is considerably smaller compared to in silico methods (Calis et al 2013; Andreatta and Nielsen 2016), it can identify post-translationally modified peptides (van der Lee et al 2019) and each peptide is actually presented by MHC-receptors (Caron et al 2015). On the downside, MS-based immunopeptidomics assay requires sufficiently large sample of tumor tissue that is not always readily available, and LC-MS/MS operation and data interpretation require expertise on the user behalf.

If the list of identified mutations is short to begin with it is possible to leave out the candidate list filtering and assay every identified neoantigen for immunogenicity. This avoids in silico prediction bias but is a practical approach for only tumors with low mutational burden. Unless one has unlimited time and resources available, which seems to be rather seldom the state of affairs in current academic research.

2.4.3 Immunogenicity

T-cells based methods are used to assess the immunogenicity of TAAs and TSAs. For TAA immunogenicity identification Rammensee depicts two types of T-cells, naïve T-cells, and pre-existing memory T-cells, and three parameters to observe the results from.

T-cell responses to a specific antigen can be tested in vitro by priming of naïve T-cells from healthy donors or by stimulating pre-existing memory T-cells in patient-derived PBMCs or TILs (Di Marco et al 2017). Pre-existing memory T-cell screening allows fast validation of T-cell epitopes, but responses can be donor dependent. Protocols used for priming naïve T-cells require co-stimulation by APCs, activated B-cells or artificial APCs (Peper and Stevanovic 2015), which can be time-consuming and costly. T-cell priming can be performed e.g. when blood samples from patients are not available.

Assay outcomes can be measured as the frequency of antigen-specific T-cells, T-cell functionality and cytotoxicity (Di Marco et al 2017). Frequency of antigen-specific T-cells can be assessed by pMHC-complex multimer staining, where combinations of fluorochrome conjugated pMHC-complexes (i.e. tetramers or pentamers) are used to bind corresponding T-cell receptors (Altman et al 1996) and number of stained T-cells counted by flow cytometry. The relatively low TCR-pMHC affinity poses a problem when applying single monomer stains, so using multimers instead resolves this issue by providing a more stable conjunction. Cytokine release of functional T-cells can be measured by intracellular cytokine staining or by enzyme-linked immunospot assay (ELISPOT). Intracellular cytokine staining recognizes T-cells internal cytokine production (Pala et al 2000), while ELISPOT is used to detect cytokine secretion (Mashishi and Gray 2002). T-cell cytotoxicity, i.e. the ability to recognize and kill tumor cells, can be measured based on chromium Cr51 release upon lysis (Brunner et al, 1968), decreased electric impedance of lysed tumor cells (Peper et al 2014) or by detecting lysosomal-associated membrane glycoproteins on the T-cell surface (Betts et al 2003).

The identified neoantigens must also be screened for immunogenicity, i.e. elicited T-cell response. First protocol to assay T-cell reactivity against neoantigen utilized target cells transfected with cancer complementary DNA libraries, recognized by cytotoxic T-cells (Coulie et al 1995). (Figure 4 depicts T-cell binding to target cell via TCR-pMHC interaction.) Although this strategy was successful in identifying several neoantigens it is also laborious, time consuming and interrogates both mutated and non-mutated peptide sequences.

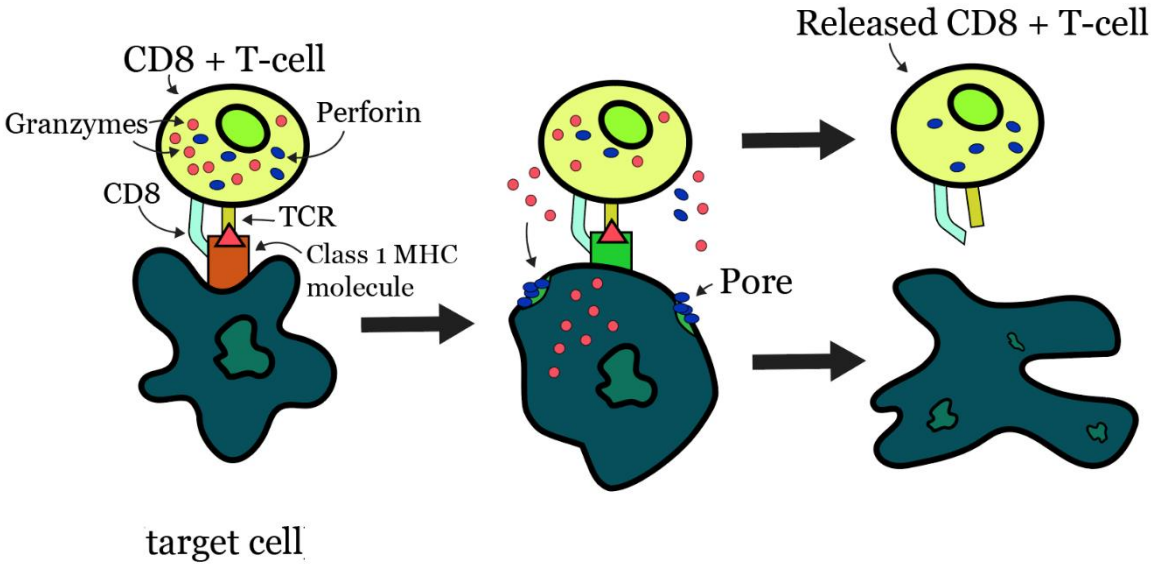

Figure 4: Cytotoxic T-cell recognizing and eliminating its target cell. TCR is interacting with target peptide presented in MHC-I/peptide complex on the cell surface, CD8 receptor stabilizing the interaction. This leads to release of perforin and pore formation on target cell, which enables granzyme to enter the cell and activate apoptosis.

Application of whole exome sequencing (See 2.4.2 Neoantigen discovery) was first introduced in 2012 (Castle et al 2012; Matsushita et al 2012). In this method the NSM derived MHC-restricted epitopes are identified from the tumor, target cells pulsed with synthesized peptides and immunological assays are employed to detect cytokine release from target recognizing TILs. This type of screening is cost-effective, but identified epitopes are limited by in silico prediction (or immunopeptidomic validation) of targets, which might incur a risk of unidentified immunogenic peptides.

Multimer staining can also be used to identify pMHC-specific T-cells. By utilizing UV-cleavable ligands a single type of pMHC allele multimers can be used to screen wide range of peptides (Toebes et al 2006; Rodenko et al 2006). However, this technique is better suited for CD8+ T-cells than CD4+ cells (Vollers and Stern 2008) and CD4+ activating neoepitopes might be difficult to identify. Also reported problems with synthetized peptide purity (de Graaf et al 2008; Currier et al 2008) might require additional screening to verify immunoreactive peptides.

In silico derived prediction bias can be avoided using tandem minigenes (TMG) (Lu et al, 2014) or peptide pools (Linnemann et al 2015) constructed from non-synonymous mutations identified in genomic analysis. APCs are transfected with TMG generated RNA or pulsed with peptide pools so that MHC-presentation pathways mimic the actual physiological process and peptides presented are verified epitopes. Afore mentioned T-cell assays are then used to confirm neoepitopes. However, when analyzing tumors with large mutational burden the methods cost effectiveness is compromised and large amounts of APCs and T-cells are required to identify neoepitopes.

Part II. EXPERIMENTAL SECTION

3 AIMS

This master thesis is a part of a project aiming to set up immunoaffinity-purification/MS-based method in order to analyze the ligandome and determine T-cell recognized cancer associated antigens from tumor cells. Objectives of the Thesis are:

- characterize several cancer cell lines for their MHC-I receptor and PD-L1 expression. Cytometric analysis of the cancer cells' MHC-I expression supports the selection of suitable cell line for the method pipeline. Additional information of the PD-L1 expression can be used to evaluate cell line's immune-evasion abilities
- collect cell material for the pMHC-purification and MS-analysis
- to explore immunological assays to determine PBMC cytokine expression (EliSPOT) and to confirm the presence of MHC-I receptors in the ligandome affinity purification eluates
- utilize in silico methods and database queries to (pseudo)confirm the presence of immunogenic MHC-I restricted peptides and to predict their immunogenic potential

4 MATERIALS AND METHODS

4.1 Reagents and equipment

Cell were cultivated in Dulbecco's Modified Eagle Medium (D-MEM), with 10% Fetal Bovine Serum (FBS, ThermoFisher Scientific) and 1% Penicillin-Streptomycin (ThermoFisher Scientific), from now on referred to as *culture medium* or *growth medium*. TrypLE™ Express Enzyme (TrypLE), Phosphate-Buffered Saline (PBS), Tween20,

formaldehyde and MilliQ grade water were used for cell detachment and the experiments. All cell culture materials were purchased from ThermoFisher Scientific.

Fluorescence readings in MTS and ELISA assays were measured with Varioskan Lux (ThermoFisher Scientific), cell counting was done with Countess (ThermoFisher Scientific) automated counter. FACS analyses were performed by BD Accuri flow cytometer (BD Biosciences).

4.2 Cell cultures

Two cancer cell lines were used in the experiments: Triple negative breast cancer (TNBC) cell line MDA-MB-436 (ATCC) and plasma cell myeloma cell line U266 (ATCC). All cell work was performed in sterile conditions under a laminar flow hood. Cultivation conditions were CO^2/temperature controlled and adjusted to 5% CO^2; 37°C. Cell cultures were constantly kept under these conditions, except for when performing experiments or dividing cell cultures for the subculture. Also, unless noted otherwise these cultivation conditions were also used for incubation of the cells during the experiments. TrypLE, a protease/EDTA solution, was used for cell detachment. Cell cultures were cultivated and counted using the following procedure: Growth medium was aspirated, and cells washed with 2,5 ml of TrypLE without incubation. 1,5 ml of TrypLE was used to incubate cells for approximately 5 minutes until the cells were detached. 8,5 ml of growth medium, pre-warmed to 37°C was added to neutralize TrypLE. Cells were counted and passaged accordingly to count results, typically at 1/5 ratio. Culture medium was renewed every 2 to 3 days.

MDA-MB-436 cells were cultured and harvested for the MS analysis. Harvesting was performed under the following protocol: Growth medium was removed and cells washed with 15 ml of 4°C PBS, aspirated without incubation. 10 ml of PBS was added, and cells detached by pipetting. Cell suspension was pipetted to a 50 ml Falcon tube and a sample gathered for the cell count. Cell suspension was centrifuged for 5 minutes at 1000G in 4°C

and cell pellet was stored in a freezer. During the harvesting all the equipment, cell culture flasks and reagents were maintained in cold conditions on an ice bed.

4.3 *In silico* methods

The online tools, The Immune Epitope Database (IEDB) and The Universal Protein Resource (UniProt) were used to analyze data derived/acquired from the MS assays. IEDB is an online database containing experimental data of more than 120 000 immune epitopes (at year 2014) associated with infectious diseases, transplantation, autoimmunity and allergies, (Vita et al 2015) but it also provides tools to predict MHC-I and MCH-II binding properties of selected peptidome and to predict the immunogenicity (T-cell epitope potential, precisely) of the peptides (Fleri et al 2017). 8-14mer peptides acquired from two datasets were analyzed by utilizing the “MCH-I binding predictions” tool (2013-02-22 version) for the MCH-I (HLA-A 01:01 and the HLA-B 08:01 alleles) binding ANN prediction method IC50 values, lower value indicating more efficient binding. The peptides with predicted IC50 values 0-1000 (predicted binders) for both HLA-A and HLA-B alleles were further analyzed by “Class I immunogenicity” tool to estimate the peptide-MHC complex immunogenicity.

The UniProt database was queried for the *homo sapiens* derived source proteins of the predicted binders and IEDB database was queried to determine if there were reference article sources that had previously identified immunogenic peptides among the predicted binders.

4.4 MTS assays

MTS cell viability assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, ProMega) was performed on MDA-MB-436 cell line, in preparation for the PBMC/cancer cell co-culture experiment. The objective was to identify suitable time frame and number of cancer cells to be seeded per well for the cell viability assay. Several concentrations and time points were applied to determine the linear range for the MTS assay.

The experiments were performed simultaneously with cell culture medium change. Cells were detached, counted and seeded on a 96-well plate under following procedure:

Growth medium was aspirated from the cell culture flask. Cells were washed with 2,5 ml of TrypLE, without incubation. Reagent was aspirated, 1,5 ml of TrypLE was added in the flash and the cells were incubated for 5 minutes. 8,5 ml of growth medium was added, and the cells were suspended for cell count and subculture. Cells were counted using Trypan blue exclusion, with live cell count of $2{,}7 \times 10^5$ cells/ml.

For the assay 5 dilutions and a blank were used, in 8 replicants. First dilution was prepared in a Falcon tube by mixing 3 ml of the initial cell suspension with 1 ml of growth medium, resulting in 4 ml of cell suspension with a concentration of 20 000 cells/100µl. 2 ml aliquot of was transferred to another Falcon tube and 2 ml of growth medium was added for the second dilution. This was repeated to prepare dilutions 1 to 5 with cell concentrations of 20 000, 10 000, 5 000, 2 500 and 1 250 cells/100µl, resp. Dilutions and the blank media were pipetted to a 96-well plate and incubated approximately 24 hours.

After incubation 20 µl of tetrazolium reagent (CellTiter 96® AQueous One Solution Reagent, ProMega) was added to each well by a multichannel pipette. The plate was incubated, and 490nm wavelength fluorescence was recorded at 1hr, 2hr, 3.5hr and 4.5hr timepoints using Varioskan Lux plate reader (ThermoFisher Scientific).

4.5 ELISPOT

Cytotoxic CD8+ T-cell response can be measured by determining their Interferon-γ (IFN-γ) production and it is commonly used to estimate T-cell activation by antigen stimulus. Here PBMC (Finnish Red Cross, Blood Service) activation was assayed using ImmunoSpot® Human IFN-γ Single-Color ELISPOT kit and CEF-Class I Peptide Pool "Plus" peptide antigen controls, both acquired from ImmunoSpot. Interleukin 2 (IL-2) induces IFN-γ production in human peripheral T-lymphocytes (Kasahara et al 1983), thus the effect of IL-2 on PBMC activation was also measured. Experiment consisted of 4 samples in duplicates; PBMC's with and without antigen stimuli, and PBMC's with and without antigen stimuli in presence of IL-2, total of 8 wells.

0.05% Tween/PBS solution, PBS and MilliQ water were used in washes, 200µl per each wash. Tween/PBS was used in to aid detachment of the cells attached during overnight incubation. All reagents were prepared *in situ* according to ELISPOT protocol. 100µl of antigen solution was used for samples with stimulus and 100µl of PBS for unstimulated samples. IL-2 was added into 4 wells. Well strip was incubated for 20 minutes. PBMC's were adjusted to 300 000 cells/100µl, plated 100µl per well and incubated for 24 hours. After incubation wash buffer solutions and anti-human IFN-γ detection solution were prepared. Plates were washed twice with PBS and once with Tween/PBS. 80µl/well IFN-γ detection solution was added and incubated for 2 hours. Tertiary solution was prepared, and wells washed three times Tween/PBS. 80µl/well of tertiary solution was added and incubated at room temperature for 30 minutes, while preparing blue developer solution. Wells were washed twice with Tween/PBS and twice with MilliQ water, 80µl/well blue developer solution was added and incubated 15 minutes in room temperature. Reaction was stopped by rinsing wells gently three times with tap water and the well strip was left to dry under a laminar hood air flow.

4.6 FACS analyses

Fluorescence activated cell sorting (FACS) flow cytometry was used to characterize MDA-MB-436 and U266 cell surfaces for HLA receptor and PD-L1 expression. For this purpose, the sample cells were stained with fluorochrome-conjugated antibodies, fixed with 4% formaldehyde/PBS-solution and analyzed with BD Accuri flow cytometer (BD Biosciences). Following protocol in three stages was used in both experiments:

I. Detachment of cells

Growth media was aspirated, and cells washed with PBS. 5 ml of 0,2% EDTA/PBS solution was added, and cells incubated until detached from the flask, approximately 5 min. 5 ml of PBS was added, cell count was performed from a sample, cell suspension was pipetted to a Falcon tube and centrifuge for 5 minutes at 1000 RPM. After centrifugation cells were resuspended with PBS to a concentration of $1{,}0x10^6$ cells/100µl.

II. Fluorochrome-antibody staining

100 µl of cell suspension per sample (1 million cells) was added to FACS staining filter cap tubes. 5 µl of Human BD Fc Block™ (BD Pharmingen) was added in each tube to eliminate unspecific antibody binding. Tubes were incubated on ice for 10 minutes without washing. 2 µl of fluorochrome-conjugated antibodies was added, tubes vortexed and incubated for 1 hour on ice in dark. Excess antibody was washed off with 1,5 ml of PBS. Tubes were centrifuged for 5 minutes in 2000 RPM and supernatant aspirated.

III. Fixing the cells

Cell pellet was resuspended with 4% formaldehyde/PBS and incubate 10 minutes at 4°C. 1 ml PBS was added, tubes were centrifuged for 5 minutes in 2000 RPM and supernatant aspirated. Cells were resuspended with 100 µl PBS for storage (up to 1 week at 4°C). For the flow cytometry analysis PBS ad 500 µl was added.

For both cell lines 5 samples were analyzed. 4 fluorophore-conjugated antibody stains, FITC Mouse Anti-Human HLA-A2 (HLA-A2 FITC); PE anti-human HLA-A2 Antibody (HLA-A2 PE); PE anti-human HLA-A,B,C Antibody (PAN-HLA PE); APC anti-human CD274 PD-L1 Antibody (PD-L1 APC), were used for staining. HLA-A1 FITC was purchased from BD Pharmingen, other antibody stains were acquired from Biolegend. Unstained cells were used to eliminate background fluorescence from the results. Also, due to technical limitations the procedure was modified for the MDA-MB-436 cell line experiment and the cells were fixed with formaldehyde BEFORE antibody staining. This is an obvious possible source of error in the experiment.

4.7 ELISA

ELISA for HLA-I was performed on ligandome affinity purification experiment eluates. MDA-MB-436 cell homogenates from two cell cultures were previously eluted thrice each through the affinity column and samples had been collected from eluates. The amount of ELISA detected HLA-I proteins is used to confirm the efficacy of the affinity column and to affirm the sufficiency of three purifications for the maximal peptide-HLA-I complex capture.

Samples (1. Immunoaffinity purification input MDA-MB-436 cell homogenate, 2. Immunoaffinity purification output cell homogenate, 3. Immunoaffinity wash, 4. 1^{st} immunoaffinity eluate fraction, 5. 2^{nd} immunoaffinity eluate fraction, 6. 3^{rd} immunoaffinity eluate fraction, 8. [sic] Concentrated, desalted and pooled eluate fractions) were analyzed with Colorimetric sandwich ELISA kit (Proteintech). 7 samples and a blank sample were analyzed in duplicates according to the following procedure: Frozen samples were thawed in room temperature for 20 minutes, centrifuged and placed on ice. Reagents and standard were prepared according to the ELISA kit instructions. Samples were diluted with sample diluent in 1:10 ratio (22 µl sample, 198 µl sample diluent). Diluted samples were kept on ice. Samples were pipetted into microplate strip wells in duplicates, 100 µl per well, and

incubated under a cover for 2 hours in 37°C. Microplate wells were washed 4 times with 300 µl of room temperature wash buffer using a multichannel pipette. 100 µl of detection antibody was pipetted in each well using a single channel pipette. Incubated for 1 hour in 37°C and washed 4 times as previously. 100 µl of HRP-conjugated antibody solution per well was added, incubated 1 hour in 37°C and washed 4 times as previously. 100 µl of TMB substrate solution per well was added in an orderly fashion. Incubated 30 minutes in dark and room temperature. 100 µl of stop solution per well in same order as TMB substrate solution was added. Samples were mixed gently, and plate read immediately using 450 nm wavelength for sample assay and 630 nm correction wavelength.

5 RESULTS

5.1 MTS assay results

MTS assay is a cell viability assay, based on tetrazolium reduction reaction (Riss et al 2013). Living cells produce NADH, which reduces tetrazolium into soluble formazan product. The amount of NADH available for reduction reaction is dependent on the number of viable cells and the fluorescence emitted by the formazan product correlates with the number of viable cells and can be measured on a plate reader.

MTS cell viability assay demonstrates good linearity for whole of the time frame (Figure 5) and each number of cells seeded per well. Results can be used to optimize the PBMC co-culture killing assay. Based on the visual inspection of Figure 6 10000 cells seeded per well might provide a workable starting point for the viability assay but other seedings within the range shown can probably be used as well. Fluorescence can also be measured on several time points at least within the 4.5-hour time period.

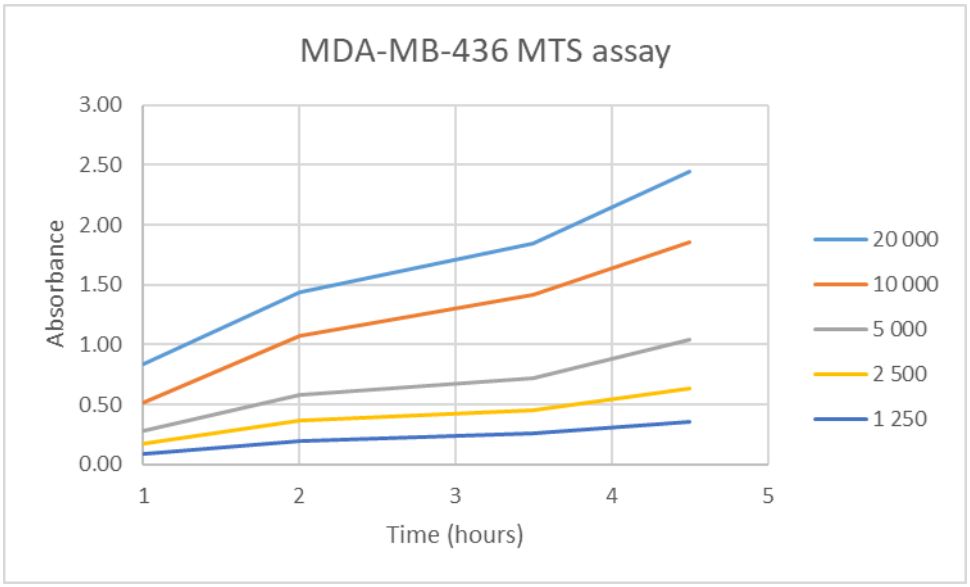

Figure 5: MDA-MB-436 MTS assay results for individual cell concentrations. All concentrations illustrate good linearity within the 4.5-hour time frame, R^2 values being more than 0.95 for all curves. (Calculated on EXCEL trendline function, not shown in the figure)

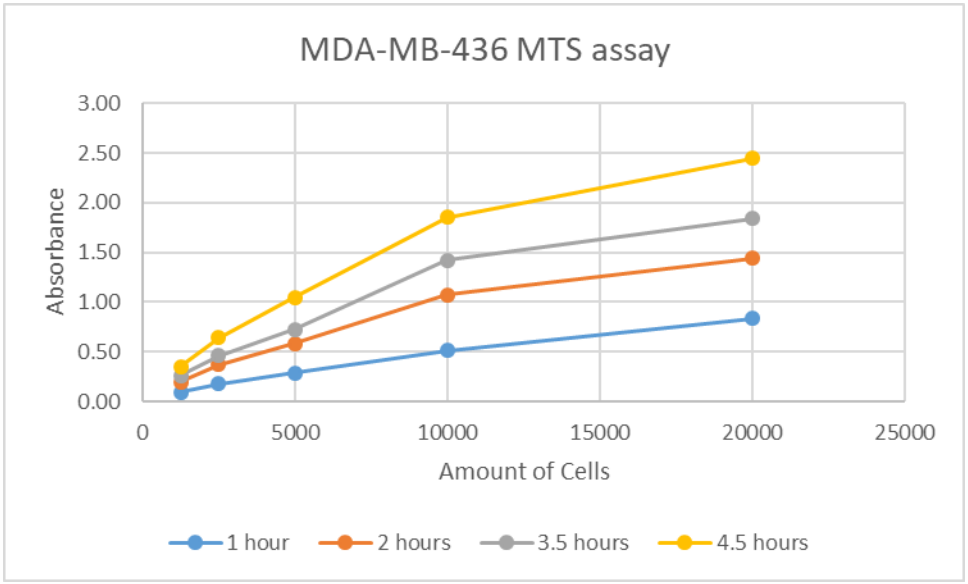

Figure 6:MTS assay results for MDA-MB-436 cell line plotted against number of cells per well. R^2 values calculated by EXCEL 1hr: 0.9892; 2hr: 0.942; 3,5hr: 0.9325; 4,5hr: 0.9368.

5.2 ELISPOT results

It was apparent from very early stage that ELISPOT results were unreadable (Figure 7) due to too large IFN-γ production from the antigen stimulated PBMC's. This was probably caused by excessive amount of PBMC's and/or antigen stimuli used for the experiment. This also rendered the evaluation of the IL-2 effect on the IFN-γ secretion impossible. Nevertheless, the results could be used adjust the number of PBMC and amount of stimuli for the future experiments.

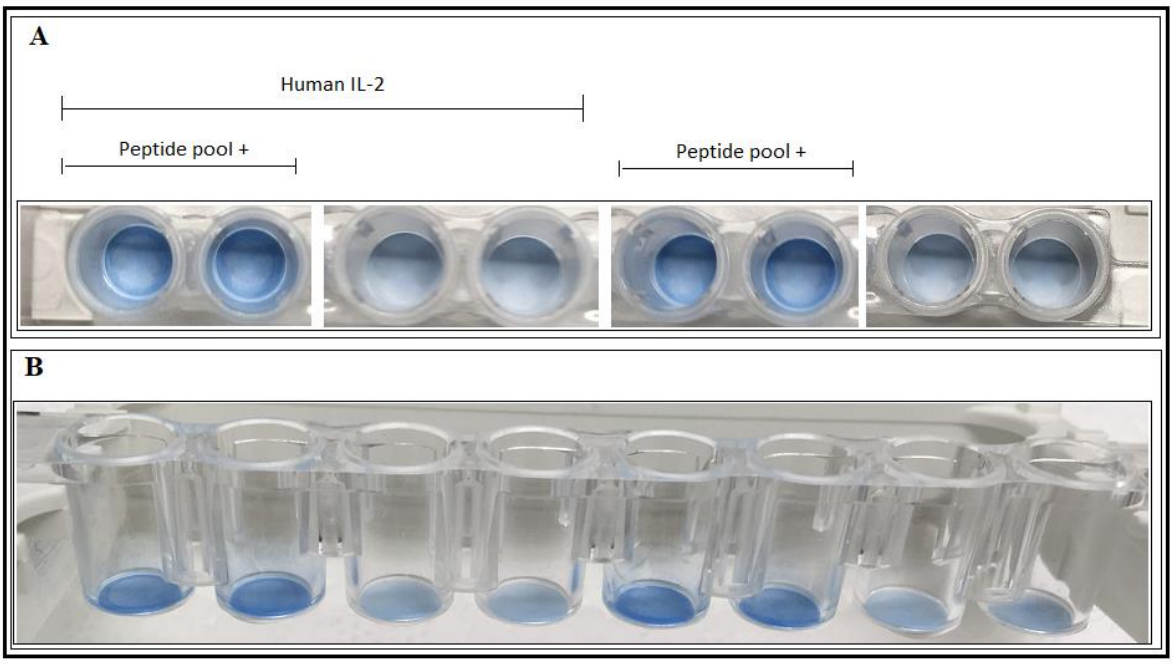

Figure 7. A: ELISPOT assay results, stimulated and unstimulated samples in presence of IL-2, and stimulated and unstimulated samples without IL-2, respectively. No individual spots can be detected from the wells with antigen stimuli. B: Wells seen from another angle to help to visualize over-colorization of samples with stimuli. Unstimulated samples exhibit no visible spotting.

5.3 FACS analysis results

FACS results (Table 1, Figure 9 and Figure 10) indicate that both MDA-MB-436 and U266 express at least some HLA allotypes (PAN-HLA PE). MDA-MB-436 cells show no sign of HLA-A2 expression, but U266 does exhibit at least some HLA-A2 expression perhaps due to some small subpopulation (Figure 8). 74% of MDA-MB-436 cells also express PD-L1, but only 1.6% of U266 cells show significant PD-L1 expression. This may also be attributed to the same subpopulation, as suggested by the histogram in Figure 10.

Table 1:Fluorescence conjugated antibody stains

MDA-MB-436							
HLA-A2 FITC		HLA-A2 PE		PAN-HLA PE		PD-L1 APC	
Cells	FITC+ %	Cells	PE+ %	Cells	PE+ %	Cells	APC+ %
262,894	0.67	256,955	0.43	237,916	75.64	162,337	74.07
U266							
HLA-A2 FITC		HLA-A2 PE		PAN-HLA PE		PD-L1 APC	
Cells	FITC+ %	Cells	PE+ %	Cells	PE+ %	Cells	APC+ %
491,548	16.1	780,202	23.48	603,736	65.32	634,167	1.65

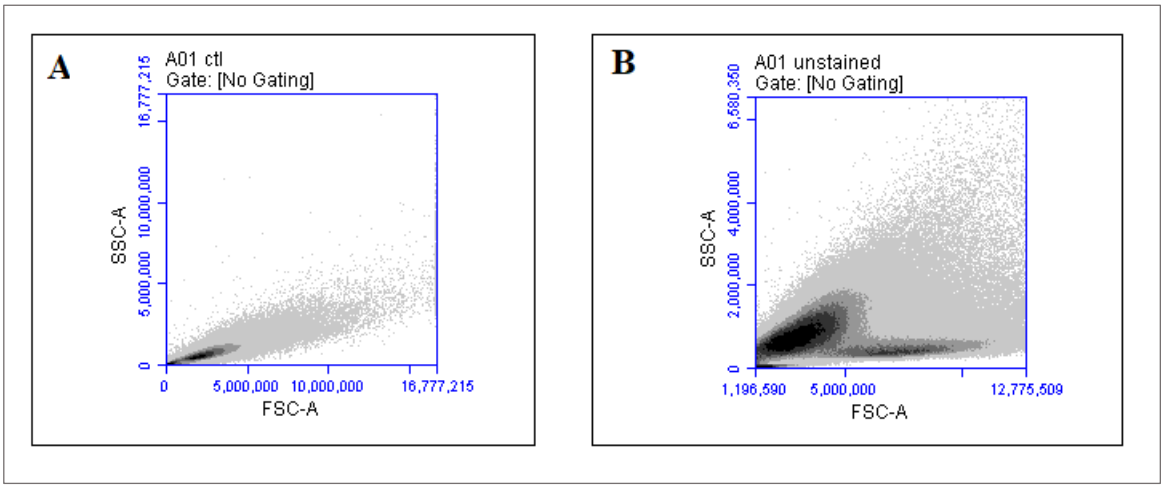

Figure 8: A: Scatter plot of MDA-MB-436 cell FACS analysis. B: Scatter plot of U266 cell FACS analysis showing two distinct cell populations.

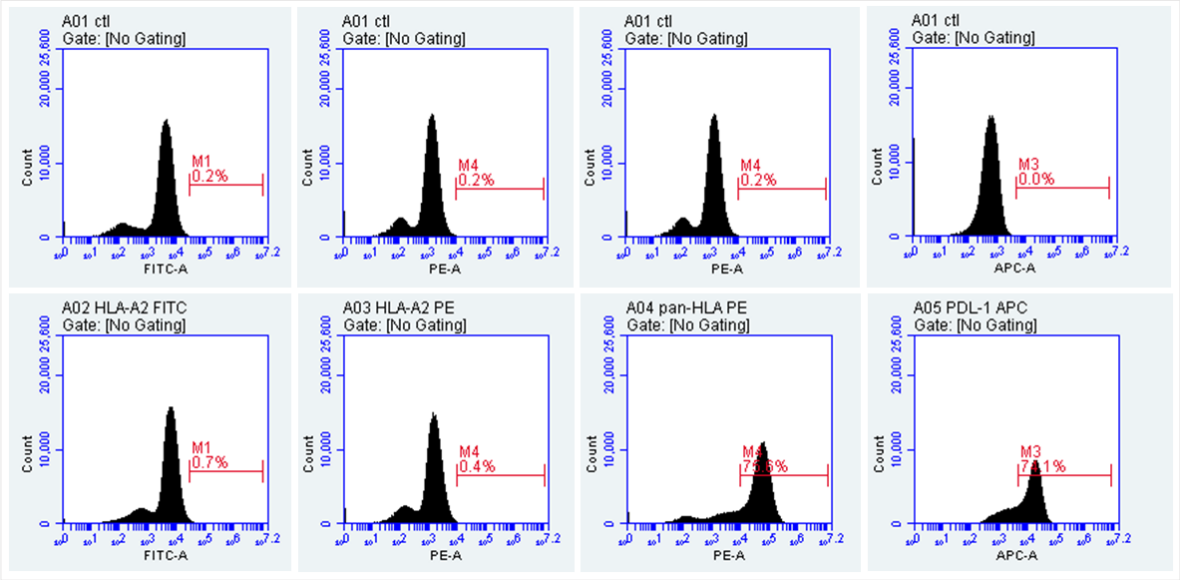

Figure 9: MDA-MB-436 Flow cytometry histograms. Upper row: Unstained cells. Lower row: Antibody stained cells left to right; HLA-A2 FITC, HLA-A2 PE, pan-HLA PE, PD-L1 APC (resp.)

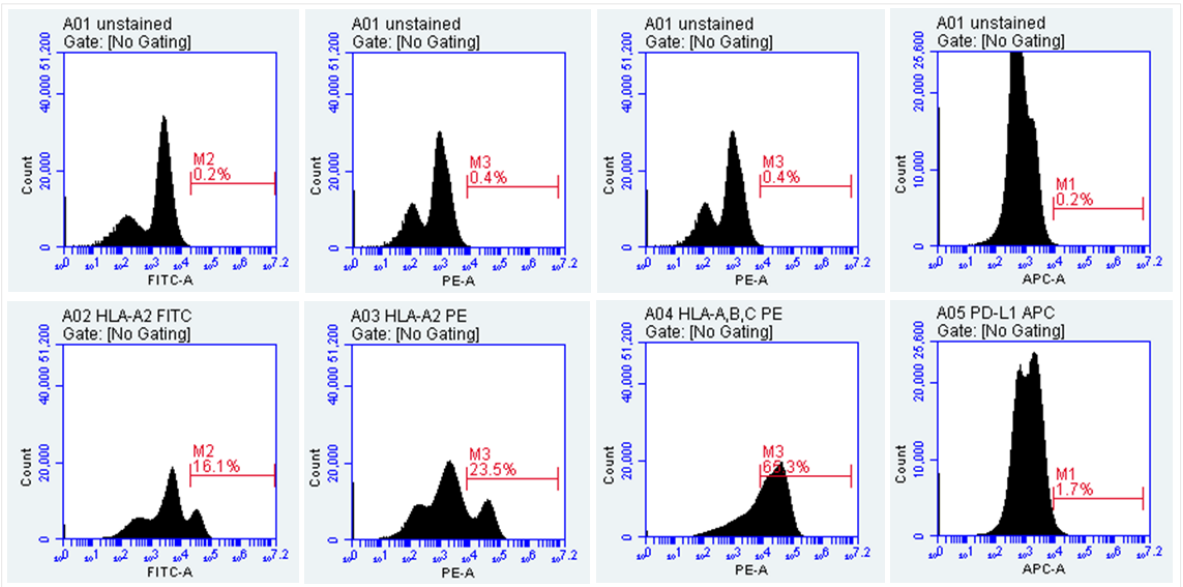

Figure 10: U266 Flow cytometry histograms. Upper row: Unstained cells. Lower row: Antibody stained cells left to right; HLA-A2 FITC, HLA-A2 PE, pan-HLA PE, PD-L1 APC (resp.)

5.4 ELISA

For each sample and standard the mean value, standard deviation and coefficient of variation (CV) was calculated. Mean values of the 630 nm correction wavelength were subtracted from the 450 nm fluorescence values, then background signal was subtracted from the samples' fluorescence by using the blank sample mean absorbance values. Standard curve was determined using EXCEL "trendline" function, best fit provided by 3rd order polynomial function, shown in Figure 11(Equation: $y = 576.62x^3 - 840.23x^2 + 1000.6x + 0.3948$, $R^2 = 0{,}9998$). For each sample CV was below 20%. Sample concentrations were solved and multiplied by 10 to compensate for 1:10 sample dilution. Results in Figure 12.

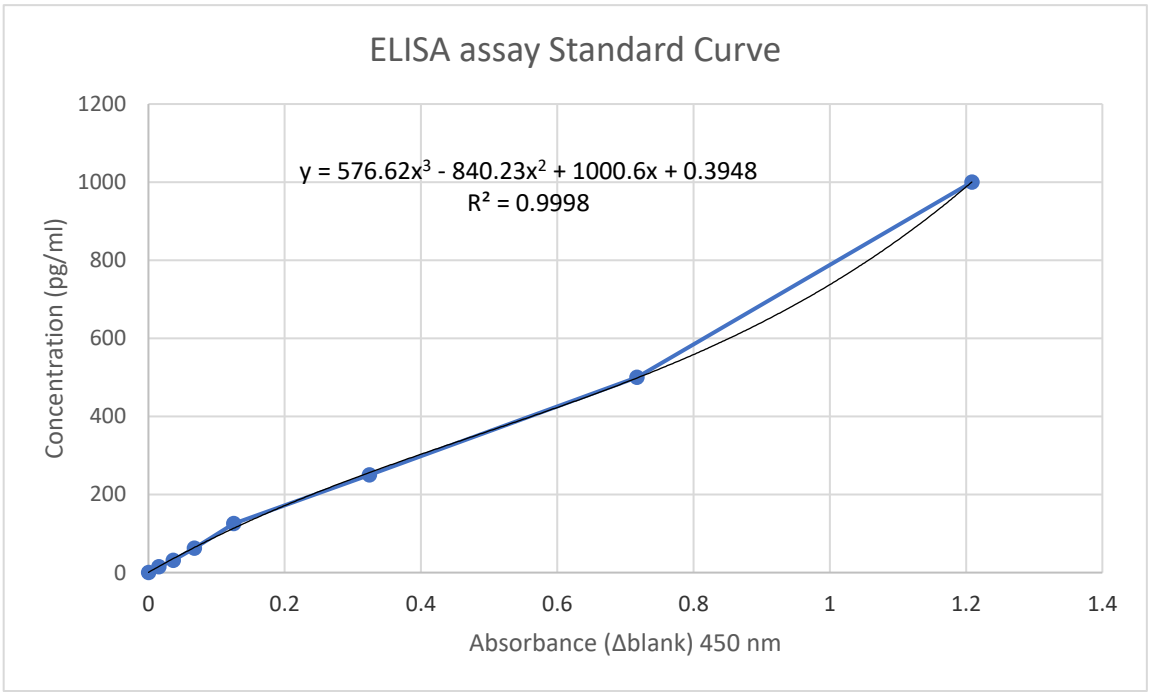

Figure 11: Standard curve with trendline equation.

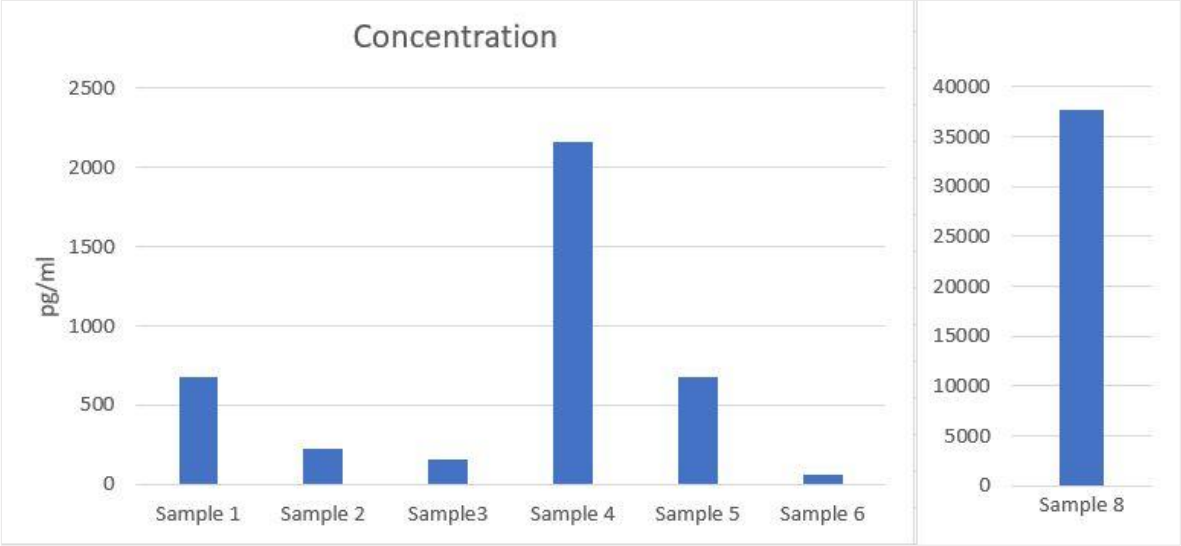

Figure 12: ELISA assay results. ***Sample 1: Cell homogenate input, Sample 2: Purification output, Sample 3: Immunoaffinity wash, Sample 4: 1st eluate fraction, Sample 5: 2nd eluate fraction, Sample 6: 3rd eluate fraction, Sample 8: Pooled, concentrated fractions.***

5.5 *In Silico* results

Two ligandome affinity purification samples analyzed by LC-MS/MS produced a total number of 96315 peptide sequences and peptide sequences length of 8-14 amino acid were selected for IEDB binding prediction analyses (Figure 13). Peptide sequence HLA binding predictions were made for HLA*A01:01 and HLA*B08:01 allotypes with ic50<1000 set as a limit for a potential ligand. This produced 199 peptide sequences as potential HLA*A01:01 binders and 467 peptide sequences as potential HLA*B08:01 binders (Figure 14 shows the size distribution of predicted MHC-restricted peptides among the analyzed data sets). IEDB Immunogenicity prediction tool was used to assess their ability to evoke T-cell response and immunogenicity score was plotted against predicted binding values to search for correlation between predicted MHC binders and T-cell epitopes. Results displayed on Figure 15 indicate no significant correlation with the ligands' predicted MHC binding ability and potential T-cell activation.

From the HLA-I A01:01 and B08:01 predicted peptide binder sets, 30 peptides of all lengths estimated to have the greatest binding ability to their corresponding HLA-receptor (ranked by ic50 values) were queried in IEDB peptide search, to learn if any had been recognized previously as actual HLA-restricted epitopes. From these 60 peptides only 3 had been recognized in other MHC-assays (Table 2). Therefor as the typical length of MHC-restricted peptide is from the shorter range and since 9mer peptides were clearly enriched among the predicted binders, all of the 9mer HLA-A1 (Table 3) and HLA-B8 (Table 4) predicted binders were queried. This provided a significantly greater amount of recognized MHC-I epitopes and one peptide, KVSAVTLAY, had been shown to induce IFNg production in T-cells by Kowalewsky et al (2015).

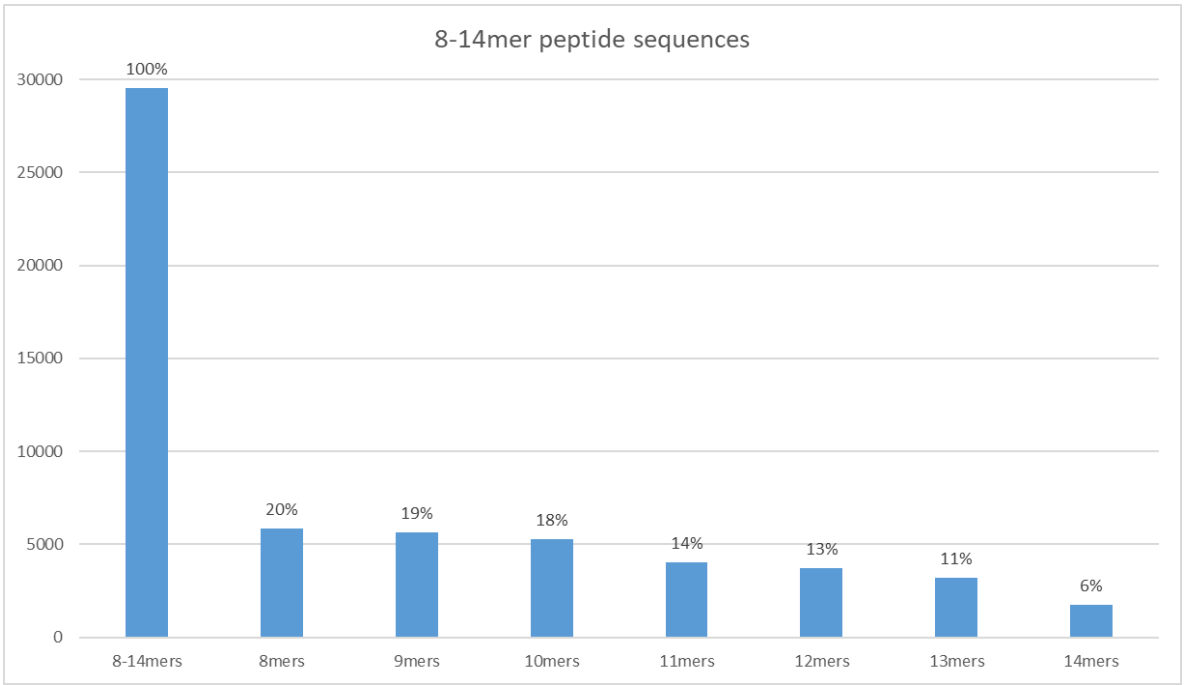

Figure 13: Total number of naturally processed ligands with peptide chain length of 8-14 amino acids. Size distribution indicates slightly higher rate of shorter peptide chains but no apparent emphasis of 9mer peptides.

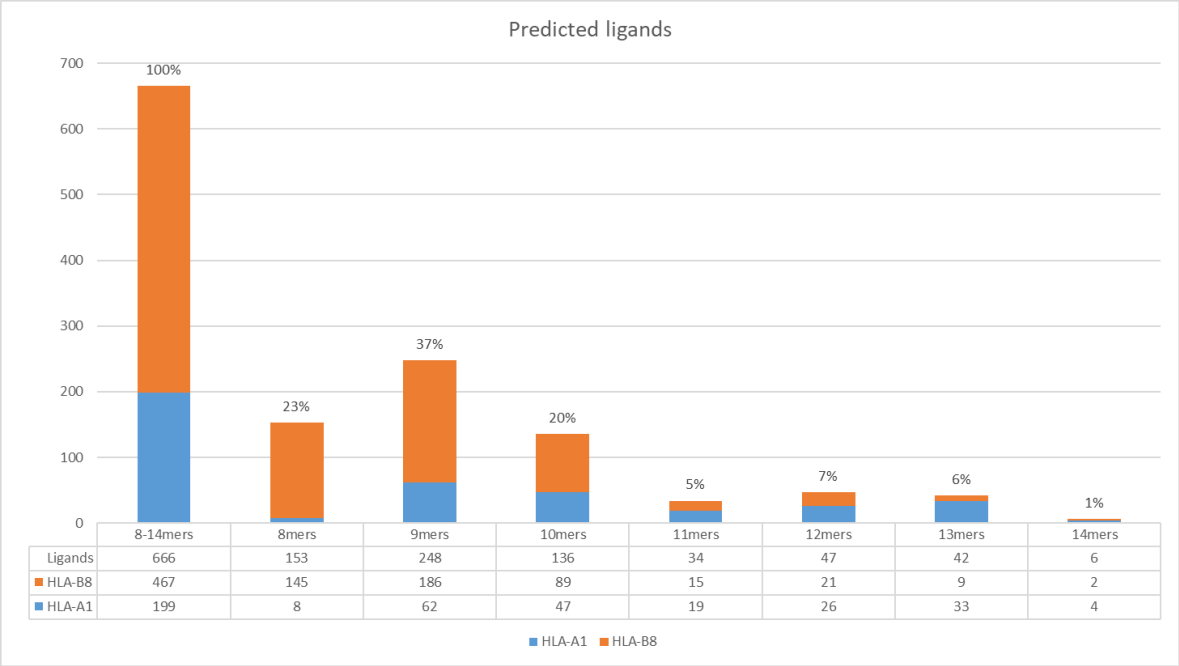

Figure 14: Predicted HLA-I allotype A01:01 and B08:01 binders (IEDB MHC-I Binding Predictions tool, ic50 value <1000). Results display a clear emphasis on 9mer peptides.

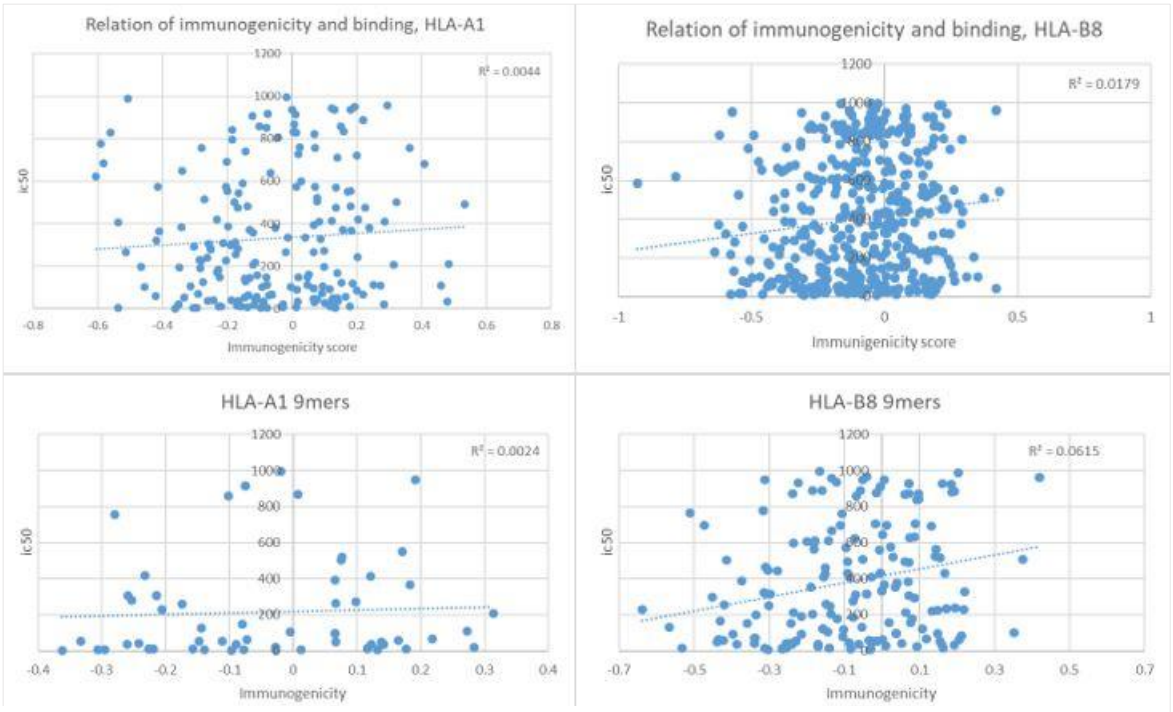

Figure 75: Predicted HLA binding ligands plotted against their predicted immunogenicity. No correlation is found between the parameters. It can also be noted that immunogenicity score values for all ligands are relatively low, 1 indicating max. immunogenicity.

Table 2: Top30 predicted immunogenic binders previously identified as MHC-I ligands. Entry refers to UniProt ID and IEDB ref gives the number of articles where the peptide has been identified in an MHC-I assay.

HLA-A1							
peptide	length	score	ic50	Entry	Protein names	Gene names	IEDB ref.
VTAIAVDRY	9	0.27254	109.56	Q9HC97	G-protein coupled receptor 35	GPR35	1
YTNRTGAVY	9	0.17682	10.89	P26006	Integrin alpha-3	ITGA3 MSK18	1
HLA-B8							
peptide	length	score	ic50	Entry	Protein names	Gene names	IEDB ref.
DAKIRIFDL	9	0.35136	102.2	Q96L21	60S ribosomal protein L10-like	RPL10L	19

Table 3: Listing of HLA-A1 9mers with known reference articles found in IEDB. Entry refers to UniProt database ID and IEDB Ref. gives the number of reference articles where the peptide has been identified as an MHC-I binder. KVSAVTLAY (marked by an asterisk) has been identified immunogenic in a T-cell assay.

Peptide seq.	score	ic50	Entry	Source Protein	Gene Name	IEDB Ref.
VTAIAVDRY	0.27254	109.56	Q9HC97	G-protein coupled receptor 35	GPR35	1
YTNRTGAVY	0.17682	10.89	P26006	Integrin alpha-3	ITGA3 MSK18	1
ATDYHVRVY	0.14164	37.03	Q53EP0	Fibronectin type III domain-containing protein 3B	FNDC3B FAD104 NS5ABP37 UNQ2421/PRO4979/PRO34274	14
YTLADGLEY	0.13841	49.68	P22033	Methylmalonyl-CoA mutase, mitochondrial	MMUT MUT	2
GTDGHVHLY	0.13248	5.12	Q96EX3	WD repeat-containing protein 34	WDR34	22
KVSAVTLAY	0.07591	521.52	P15391	B-lymphocyte antigen CD19	CD19	12*
VTSGKIIEY	0.0665	265.84	P51610	Host cell factor 1	HCFC1 HCF1 HFC1	7
VSDNDIRKY	0.06525	394.11	P55072	Transitional endoplasmic reticulum ATPase	VCP	10
ALAGGMFLY	0.00682	867.05	Q9C0K1	Zinc transporter ZIP8	SLC39A8 BIGM103 ZIP8 PP3105	1
STDPRPASY	-0.02764	7.29	O15169	Axin-1	AXIN1 AXIN	17
VTEIDQDKY	-0.0283	20.72	P21333	Filamin-A	FLNA FLN FLN1	26
SSEAHLQQY	-0.07251	62.76	P48552	Nuclear receptor-interacting protein 1	NRIP1	7
LTEQYNEQY	-0.07693	5.75	Q9BWF3	RNA-binding protein 4	RBM4 RBM4A	20
SSDPYHSGY	-0.09693	4.48	Q9BXP5	Serrate RNA effector molecule homolog	SRRT ARS2 ASR2	12
LSAVLKDLY	-0.14732	52.37	Q9C0B7	Transport and Golgi organization protein 6 homolog	TANGO6 KIAA1746 TMCO7	1
KTDESLTKY	-0.15683	11.63	Q9HCK8	Chromodomain-helicase-DNA-binding protein 8	CHD8 HELSNF1 KIAA1564	12
YTSPGTQKY	-0.21908	10.22	Q6P1X5	Transcription initiation factor TFIID subunit 2	TAF2 CIF150 TAF2B	11
ISDPLQQCF	-0.25306	280.26	Q15650	Activating signal cointegrator 1	TRIP4	1
PSDPKQYRY	-0.29588	6.02	O43847	Nardilysin	NRDC NRD1	20
TSDYVSQSY	-0.30647	5.09	Q96K76	Ubiquitin carboxyl-terminal hydrolase 47	USP47	8
FTDKKTHLY	-0.36244	2.06	P27824	Calnexin (IP90)	CANX	16

Table 4: Listing of HLA-B8 9mers with known reference articles found in IEDB. Entry refers to UniProt database id and IEDB Ref. gives the number of reference articles where the peptide has been identified as an MHC-I binder.

Peptide seq.	score	ic50	Entry	Source Protein	Gene Names	IEDB ref.
AIKRKHAVL	-0.14033	14.14	Q96AE7	Tetratricopeptide repeat protein 17	TTC17	1
DAKIRIFDL	0.35136	102.2	Q96L21	60S ribosomal protein L10-like	RPL10L	19
DAKSKIRAL	-0.25465	41.94	Q92598	Heat shock protein 105 kDa	HSPH1 HSP105 HSP110 KIAA0201	9
DVKGRIYQL	0.06898	385.69	Q15021	Condensin complex subunit 1	NCAPD2 CAPD2 CNAP1 KIAA0159	6
DVKSKTEAL	-0.30257	446.42	P53618	Coatomer subunit beta	COPB1 COPB MSTP026	7
EAKQRLQQL	-0.31204	15.94	Q9P0U1	Mitochondrial import receptor subunit TOM7 homolog	TOMM7 TOM7 TOMM07 AD-014	10
EIKRINKEL	-0.01791	706.89	O94973	AP-2 complex subunit alpha-2	AP2A2 ADTAB CLAPA2 HIP9 HYPJ KIAA0899	6
EIKTKIKEL	-0.23916	36.44	Q92598	Heat shock protein 105 kDa	HSPH1 HSP105, HSP110, KIAA0201	6
ELHKKLLSV	-0.53296	17.58	Q96F86	Enhancer of mRNA-decapping protein 3	EDC3 LSM16 YJDC YJEFN2 PP844	10
ELKIKRSLF	-0.24346	39.69	Q9NVA2	Septin-11	SEPT11	5
ELKTKIEKL	-0.15716	123.8	Q96EA4	Protein Spindly	SPDL1 CCDC99	2
ELLKRKEAL	-0.26584	12.73	Q8N556	Actin filament-associated protein 1	AFAP1 AFAP	2
FLKRKEEEL	0.00933	29.88	Q68D10	Protein SPT2 homolog	SPTY2D1	2
HVGSRLYSV	-0.21529	191.05	P21333	Filamin-A	FLNA FLN FLN1	3
IVKPKPRAL	-0.23506	213.37	Q92539	Phosphatidate phosphatase LPIN2	LPIN2 KIAA0249	7
KLHQRVEQL	0.00002	331.51	P49716	CCAAT/enhancer-binding protein delta	CEBPD	6
KLLLRLPAL	0.0387	95.32	P19793	Retinoic acid receptor RXR-alpha	RXRA NR2B1	1
LAAARLAAA	0.14791	224.77	P30101	Protein disulfide-isomerase A3	PDIA3 ERP57 ERP60 GRP58	16
NGATRKLAL	-0.08734	46.53	P31689	DnaJ homolog subfamily A member 1	DNAJA1 DNAJ2 HDJ2 HSJ2 HSPF4	12
QIKQKVDSL	-0.43564	61.29	O60812	Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1 HNRPCL1	4
QLKHKLEQL	-0.24107	152.16	P01106	Myc proto-oncogene protein	MYC BHLHE39	3
QVIEKTKSL	-0.30817	316.97	P60228	Eukaryotic translation initiation factor 3 subunit E	EIF3E EIF3S6 INT6	6
RVKKKNEAL	-0.39573	94.68	O95696	Bromodomain-containing protein 1	BRD1 BRL BRPF2	4
SPLGLVLAL	0.07206	929.08	O00562	Membrane-associated phosphatidylinositol transfer protein 1	PITPNM1 DRES9 NIR2 PITPNM	2
VIKSRYQTL	-0.26463	13.98	Q8WVK7	Spindle and kinetochore-associated protein 2	SKA2 FAM33A	8
WVKEKVVAL	-0.08269	12.83	P24001	Interleukin-32	IL32 NK4 TAIF	7
YARLHPRAV	0.09324	25.9	P62987	Ubiquitin-60S ribosomal protein L40	UBA52 UBCEP2	11

6 DISCUSSION

The advances in immunotherapies are opening possibilities in the treatment of cancer, infections and autoimmune disease, but also made it imperative to develop methods to characterize individual immunopeptidome associated with aforementioned conditions. The accurate immunopeptidome profiling is essential to provide personalized and effective treatments and to reduce related adverse reactions. The project this thesis is a part of is

dedicated in the discovery of validated immunogenic T-cell epitope, that can be utilized e.g. in personalized cancer vaccines, immunovirotherapy and/or cell-based treatments. The project's antigen discovery workflow consists of two parallel pipelines, the other focusing on tumor immunopeptidome discovery and the other on tumor neoepitome discovery. Neoantigen discovery, even if briefly introduced in the literature review section, involves methods such as genetic sequencing and is beyond the scope of this thesis, but the experiments conducted and presented in the experimental section can be used as a part of setup for tumor immunopeptidome discovery pipeline.

The immunopeptidome discovery pipeline consists of gathering tumor cell material for the immunoaffinity purification, optimizing the immunoaffinity process, profiling the MHC-ligands from the acquired samples by LC-MS/MS (outsourced and not described in this work) and interrogating public databases (e.g. UniPROT and IEDB) to identify immunogenic epitopes. Both the immunopeptidome and neoepitome need to be validated (by LC-MS/MS) and analyzed for their immunogenicity. Immunogenicity of the peptides can be analyzed by T-cell assays such as multimer staining and T-cell/cancer cell co-culture killing assays. Experiment performed for this thesis can assist in the selection of suitable cancer cell line (FACS analysis, MTS assay), optimizing cMHC-complex purification process (ELISA), development of T-cell activity assays (EliSPOT and MTS assays) and to screen for potential immunogenic peptides from MS data (database queries). The experiments and results are explored.

Preliminary MTS assay successfully determined the usable range and time limits for further assays. This is useful information regarding the co-culture killing assay, which determines the efficacy of selected epitope antigen peptide recognizing PBMCs' in killing the cancer cells. Anyway, it is recommendable to conduct more precursory MTS assays with both PBMCs and cancer cells to assess the influence of co-culture on the assay linearity, reproducibility, and sensitivity.

FACS analysis provided ambiguous results. Both of the analyzed cell lines (U266 and MBA-MD-436) expressed HLA alleles, as indicated by the PAN-HLA PE immunostaining. Approximately 75% of MBA-MD-436 cells expressed HLA-receptors, as did 65% of the U266 cells. It is possible that the rest of the cells did not express HLA-receptors, perhaps because of developing HLA-deficiency through mutation, but since the cell cultures didn't experience evolutive pressure by immune system driving the cells to lose HLA expression a more plausible explanation for the diversity could be the technical deviation from the experiment protocol. I.e., the staining of the cells simply was not very successful. Whatever the reason might be, at least majority of the cells had HLA expression, indicating suitability for the peptide-HLA complex purification. But U266 cells' two distinct populations (Figure 8, figure 10) and limited HLA-A2 staining raised questions, because it could be expected that the cells' HLA-expression profile would've been more consistent. Also, the PD-L1 expression in MBA-MD-436 cells was interesting, since it implied that the cells might be able to avoid apoptosis by tumor selective T-cells. Utilizing PD-L1 expressing cancer cells in experiments could be used e.g. to probe the effect of using immunocheckpoint inhibition in tandem with other immunotherapies.

Subsequently, the U266 cells were examined by HLA typing, and it was revealed that the cell culture had indeed been cross contaminated, apparently by OPM-2 cells (Unpublished data). Curiously, this was not the reason for the separate cell populations shown in scatter plot in Figure 8. Nevertheless, the U266 cell line was not used further for research purposes.

The ELISA results confirm the presence of the HLA-I (Human MHC-I) complex in the MDA-MB-436 cell lysates. Sample 1 presents the HLA-I concentration in the actual homogenized cell material, while the sample 2 presents the concentration of HLA-I in the immunoaffinity purification column output. The difference in Sample 1 and Sample 2 concentrations is a direct indication of the immunoaffinity column efficacy: the greater the difference in the input and output concentrations, the better the immunoaffinity column binding. Sample 3 shows the concentration of HLA-I in the column wash product. Ideally

this signal should be comparatively low since the wash should remove only unspecific bound material instead of HLA-I complex. The desirable result would thus present a significant subtraction in concentration from Sample 1 to Sample 2, with as low as possible detection of HLA-I in Sample 3, because in that case the immunoaffinity column would be shown to bind effectively HLA-I complexes during the purification, with no unbound complexes present in the wash. The results of the ELISA assay indicate that the binding capacity of the immunoaffinity column is not necessarily as good as desired, since evidently a lot of target material couldn't be captured in the purification, and significant amount of (potentially immunogenic) HLA-restricted peptides are lost. In this case the column efficacy should be enhanced e.g. by adjusting the amount of HLA-I binding antibodies in the purification column.

After the purification column wash the HLA-I complexes are recovered (i.e. eluted) from the HLA-I binding antibodies by using suitable buffer solution that disrupts the affinity interaction. Concentration in Sample 4 is considerably greater than in the input cell lysate in Sample 1. This is explained because the HLA-I is already concentrated in the elution process. How much the HLA-I is present in the eluate fractions indicates how much of the target molecules are regained from the immunoaffinity purification. Since the Sample 6 (3rd eluate fraction) shows detectable amount of HLA-I the number of fractions could be increased to make sure that the target molecules are recaptured as efficiently as possible. It should be noted though, that Sample 7 was present in the ELISA assay setup but could not be analyzed because lack of reagents. The amount of HLA-I in Sample 7 was estimated to be quite low so it was decided to leave unanalyzed. Retrospectively it would have granted information of the elution efficacy. The Sample 8 is of the concentrated and pooled eluate, that did not deliver linear results with other samples. In fact, it was expected to go out of assay range and to confirm that the results from other samples were meaningful and produces coherent signals.

Samples derived from the immunoaffinity purification were analyzed by LC-MS/MS (outsourced analysis). Regardless of the current breakthrough of *in silico* prediction

methods for MHC-binding neoantigens the MS is the only reliable method that can produce unbiased results for naturally presented, possibly post-translationally edited immunoepitopes (Bassani-Sternberg and Coukos 2016) and can be used to determine immunopeptidome of cell lines, tumor tissue, healthy tissue and body fluids. Still, even though immunopeptidome analysis by MS presents regularly typical size distribution and binding motifs of MHC-restricted peptides, and even if the method has been successfully used to recognized several tumor-specific antigens the results have to be validated and acquired peptides evaluated for their immunogenicity. This sort of validation and testing is out of scope of this Master's Thesis, but the datasets were analyzed and "pseudo-confirmed" by investigating the predicted HLA-binding potential and immunogenicity by *in silico* tools provided by IEDB database. The binding ability of peptides in datasets were interrogated, peptides ranked by their ic50 value and a cut-off value of ic50<1000 was utilized to determine probable HLA-binders. Previous studies have applied different cut-off values such as ic50<500, but ic50<1000 was deemed suitable to gather as much potential epitopes as possible.

MHC-I restriction (binding) is essential to induce the T-cell mediated immune response, but a strong restriction is not equivalent with a strong immunogenic response from the T-cells. The IEDB represents the peptides tested for MHC binding, MHC ligand elution or T-cell recognition, and the details of the experimental contexts in which these molecules were tested (Kim et al 2011), but nonetheless these tools can provide only an algorithm based estimate of the peptide characteristics. Only peptides that test positive in a T-cell recognition assays are truly 'T-cell epitopes', while those that are only shown to be positive in a binding assay should be referred to as 'MHC binders' and those that can be eluted from MHC on the cells surface are "naturally processed ligands". Several methods are available for the peptide's MCH restriction capability prediction, but Artificial Neural Network (ANN) method has outperformed other methods regarding the MCH-I binding prediction and was employed for the analysis. When comparing the predicted binders with the predicted immunogenicity it was apparent that there was no correlation, at least within this

data set, and that there were no apparent strong candidates for actual T-cell epitopes. Naturally, as described earlier, a significant amount of HLA epitopes were probably lost in the purification process and among the missing peptides there might have been potential immunogenic tumor-associated antigens. Of course, when screening for potential antigen targets to be used in actual therapy it would be desirable to locate tumor antigens with considerable expression throughout the tumor tissue; Tumor antigens not widely expressed in tumor tissue would make poor targets for immunotherapy.

The peptides were subsequently ranked by predicted immunogenicity. Of this set the 30 peptides with best immunogenicity score were selected for IEDB peptide search to screen for HLA-ligands discovered previously in other assays. Of these peptides, not vetted by size, only 3 had been recognized previously as HLA-binders. Considering that typical MHC-restricted peptides are length of 8-10 amino acids, the 9mers that were clearly enriched in the binding prediction analysis were screened. Peptides the length of 9 amino acids were selected because they were clearly emphasized in predicted MHC-binders, and because the number of peptides was still manageable for the manual screening. This set provided numerous peptides recognized previously (see Results) with one peptide recognized immunogenic in a T-cell assay. It was estimated from this result, that the method can potentially be used to discover immunogenic peptides from the cell culture.

Even though the results can be interpreted as positive and IEDB tools for predicted MHC-restriction and immunogenicity could supposedly be used to characterize immunopeptidome of the cells, it must be recognized that this method of validation is flawed at best. The predicted peptides should ideally be synthesized and reanalyzed by MS to confirm the MS fingerprint of the peptides and validated peptides assessed by T-cell assay to confirm immunogenicity, because of the uncertainty related to algorithmic prediction methods (Garcia-Garijo et al 2019). Also, compared to database query a more reliable method to confirm the epitopes would be comparison to data sets from the applied cell line and/or RNA-analysis of the cells to determine if the peptides' source proteins are actually being expressed. Moreover, it is unlikely that the method can be used to identify actual immunogenic

neoantigens, because the *in silico* analysis of predicted epitopes is based on algorithms trained with existing datasets and their assumptions on binding/immunogenicity characteristics. In this sense the method can be considered to be based in "historically" identified epitopes instead of being able to acquire novel information on antigens. The risk of reinforcement bias is also present. If the datasets are created and peptides characterized by *in silico* predictions and these are in turn used to train the algorithms it is easy to perceive the possibility of self-amplifying cycle, that can potentially lead to omission of immunogenic peptides. Nonetheless, it must be considered that this experiment is only a minor part of larger project and does not as such reflect the whole immunoligandome discovery pipeline. Even if the method at this stage can be considered only nominally validated the results can be applied in the further development of the project.

7 CONCLUSIONS

The research for immunopeptidome discovery methods is an essential part of applications for modern personalized immunotherapies. Successful identification of tumor-associated and tumor-specific antigens enables the development of treatments with greater efficacy and reduced adverse effects.

Even if the execution and the results from experiments described above were not completely unproblematic, they managed to produce information that could be used to develop the assays further. *In silico* methods were successfully used to characterize previously identified HLA-restricted peptides and one previously identified immunogenic T-cell epitope. Even if the method cannot be considered adequately validated the results encourage the further development of the method, taking into account the methods limitations and the requirement for appropriate validation.

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